"Ideas must work through the brains and arms of men,

or they are no better than dreams."

*Ralph Waldo Emerson*

**University of Alberta**

# Motor units in the SOD1$^{G93A}$ transgenic mouse model of ALS

by

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## ABSTRACT

Motoneuron dysfunction precedes the overt clinical disease in both patients with Amyotrophic Lateral Sclerosis (ALS) and in the SOD1$^{G93A}$ mouse model of ALS. In agreement with anatomical studies, we showed that in 60 day old SOD1$^{G93A}$ mice, ~1 month before the onset of symptoms, ~60% of the functional motor units in the fast-twitch tibialis anterior (TA) muscle of the SOD1$^{G93A}$ mouse are already lost. Although the average number of fibers innervated per motoneuron (the innervation ratio; IR) was maintained, the surviving motor units were less forceful. Dissociation between motor unit force and size has previously been found only in symptomatic ALS patients. Here, we are the first to show that the dissocation was due to an increase in the proportion of the less forceful type IIA and IID/X muscle fibers. Both preferential denervation of the most forceful type IIB muscle fibers and the activity-dependent conversion of innervated muscle fibers contributed to the change in muscle fiber type proportions. Fast-twitch muscles, which had the greatest content of type IIB muscle fibers were consistently more vulnerable to motor unit loss across the entire lifespan of the SOD1$^{G93A}$ mouse. There was an early and selective decline in the number of intact motor units in the fast-twitch TA, extensor digitorum longus (EDL) and medial gastrocnemius (MG) muscles, evident as early as 40 days of age, 50 days before the reported onset of overt symptoms and coincident motoneuron loss. In the slow-twitch soleus muscle, contractile forces and motor unit numbers did not consistently decline until 90-days of age. The early and preferential, gender-independent vulnerability of motor units in fast-twitch muscles led us to hypothesize that daily recruitment patterns and associated differences in muscle contractile properties dictate susceptibility in SOD1$^{G93A}$ mice. In order to test the hypothesis the recruitment of surviving motor units was next increased by avulsion of one of the spinal roots supplying the hindlimbs. In agreement with our hypothesis motor unit loss was attenuated by root avulsion. In conclusion, our finding that increased activity confers protection to motor units in the SOD1$^{G93A}$ mouse has implications for both the diagnosis and treatment of ALS.

## ACKNOWLEDGEMENTS

"Research is four things: brains with which to think, eyes with which to see, machines with which to measure and, fourth, money."

*Albert Szent-Györgyi, Hungarian physiologist and Nobel Laureate*

As suggested by my compatriot, Szent-Gyorgyi, multiple brains are needed for research. Indeed, I could not have completed my doctoral studies without the help of the many exceptional "brains" whom I now wish to acknowledge. First, I would like to thank my supervisor and mentor, Dr Tessa Gordon, who welcomed me into her laboratory almost five years ago. From the beginning, I was allowed a measure of independence in the laboratory that I never expected, but which I quickly grew to appreciate. Tessa always trusted my judgment and taught me to believe in myself.

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*For my mom and dad, whom I could never disappoint,*

*and*

*for my grandparents, who encouraged me to learn.*

*"Légy okos"*

## TABLE OF CONTENTS


**CHAPTER 1: INTRODUCTION** 1

1.1 MOTOR UNITS: BUILDING BLOCKS OF THE NEUROMUSCULAR SYSTEM

1.1.1 Anatomy and physiology of the motor unit 2

1.1.2 Intrinsic motoneuron properties and the Size Principle 4

1.1.3 The muscle fibers of the motor unit 6

1.1.4 Changing motor unit activity causes adaptations in both motoneurons and the muscle fiber properties 9

1.1.5 Partial denervation and motor unit adaptations 13

1.1.6 Motor unit number estimation 16

1.2 COMPENSATORY MOTOR UNIT CHANGES IN AMYOTROPHIC LATERAL SCLEROSIS (ALS) PATIENTS

1.2.1 Amyotrophic Lateral Sclerosis (ALS) 19

1.2.2 Diagnosis of ALS 21

1.2.3 Prognosis and treatment of ALS 22

1.2.4 Pathophysiology of upper motoneuron loss in ALS 23

1.2.5 Physiology of motor units in ALS 24

1.3 MOTOR UNITS IN ANIMAL ALS MODELS

1.3.1 Animal models of ALS 27

1.3.2 The $SOD1^{G93A}$ mouse model of ALS 30

1.3.3 Motoneuron pathology in the $SOD1^{G93A}$ transgenic mouse 32

1.3.4 Effects of gender on the $SOD1^{G93A}$ mouse model of ALS 33

1.3.5 Exercise effects disease progression $SOD1^{G93A}$ mouse model of ALS 34

1.3.6 Evidence of motor unit loss in the $SOD1^{G93A}$ mouse model 36

1.4 THE OBJECTIVES AND AIMS OF THE PHD THESIS 38

1.5 BIBLIOGRAPHY FOR CHAPTER 1 46

**CHAPTER 2: SELECTIVE LOSS OF THE MOST FORCEFUL MOTOR UNITS IN THE PRE-SYMPTOMATIC $SOD1^{G93A}$ MOUSE MODEL OF ALS** 68

2.1 INTRODUCTION 68

2.2 METHODS

2.2.1 $SOD1^{G93A}$ and $SOD1^{WT}$ mice 71

2.2.2 Electrophysiological studies 72

2.2.3 Muscle fiber type determination for TA muscle 74

2.2.4 Statistical analysis 76
2.3 RESULTS
2.3.1 Motor unit force declines in the TA muscle of SOD1$^{G93A}$ mice before the onset of overt weakness 77
2.3.2 The parallel decline in average motor unit force and number caused a reduction in whole muscle force in the pre-clinical phase of disease in SOD1$^{G93A}$ mice 79
2.3.3 Motor unit force declines due to a decrease in the force produced per muscle fiber 80
2.3.4 Changing muscle fiber type proportions causes a shift in CSA distribution 82
2.3.5 Preferential denervation causes a decrease in muscle fiber CSA 85
2.3.6 Preferential functional motor unit loss in an antagonistic muscle 86
2.4 DISCUSSION 88
2.5 BIBLIOGRAPHY FOR CHAPTER 2 104

**CHAPTER 3: TIME COURSE OF PREFERENTIAL MOTOR UNIT LOSS IN THE SOD1$^{G93A}$ MOUSE MODEL OF AMYTOTROPHIC LATERAL SCLEROSIS (ALS) 110**

3.1 INTRODUCTION 110
3.2 METHODS
3.2.1 Generation of SOD1$^{G93A}$ mice 113
3.2.2 Electrophysiological studies 114
3.2.3 Myosin heavy chain determination 117
3.2.4 Statistical analysis 118
3.3 RESULTS
3.3.1 Comparison of SOD1$^{WT}$ and non-transgenic control mice 119
3.3.2 Phenotypic composition of four hindlimb muscles 119
3.3.3 Selective muscle susceptibility during the pre-clinical phase of disease in the SOD1$^{G93A}$ mice 121
3.3.4 Selective motor unit loss at earliest signs of disease pathology 122
3.3.5 Muscle phenotype dictates susceptibility to motor unit loss 124
3.3.6 Contractile force declines in parallel with motor unit numbers 126
3.4 DISCUSSION 128
3.5 BIBLIOGRAPHY FOR CHAPTER 3 143

**CHAPTER 4: THE EFFECTS OF GENDER ON PROGRESSIVE MOTOR UNIT LOSS IN A TRANSGENIC MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS (ALS)** 149

4.1 INTRODUCTION 149

4.2 METHODS

4.2.1 Generation of $SOD1^{G93A}$ mice 150

4.2.2 Electrophysiological recordings 150

4.2.3 Statistical analysis 151

4.3 RESULTS

4.3.1 Motor unit number 152

4.3.2 Muscle contractile force 153

4.4 DISCUSSION 155

4.5 BIBLIOGRAPHY FOR CHAPTER 4 161

**CHAPTER 5: FUNCTIONAL OVER-LOAD SAVES MOTOR UNITS IN THE SOD1-G93A TRANSGENIC MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS (ALS)** 165

5.1 INTRODUCTION 165

5.2 METHODS

5.2.1 Generation of $SOD1^{G93A}$ mice 168

5.2.2 Voluntary wheel running 168

5.2.3 Partial denervation of hindlimb muscles 169

5.2.4 Electrophysiological recordings 170

5.2.5 Statistical analysis 171

5.3 RESULTS

5.3.1 Preferential motor unit loss in $SOD1^{G93A}$ mice around the time of symptom onset 172

5.3.2 Voluntary wheel running has gender dependent effects 173

5.3.3 Wheel running accelerates the loss of motor units in TA muscles of male $SOD1^{G93A}$ mice 174

5.3.4 Wheel running saves motor units in the MG muscles of female $SOD1^{G93A}$ mice 175

5.3.5 The effects of voluntary wheel running depend on gender in $SOD1^{G93A}$ mice 177

5.3.6 Partial denervation reduces the number of functional motor units in the TA muscle 178

5.3.7 Partial denervation is also compensated for in the other hindlimb muscles of WT control mice 180

5.3.7 Whole muscle twitch force is maintained in the $SOD1^{G93A}$ mice following avulsion of L4 ventral root 181

5.3.8 Motor units do not become more forceful to compensate for partial denervation in the $SOD1^{G93A}$ mice 182

5.3.9 Avulsion of L4 ventral root confers protection upon the remaining motor untis in the $SOD1^{G93A}$ male mice 184

5.3.10 Motor units are also saved after avulsion of L5 root 186

5.3.11 Protection of motor units following avulsion of L4 ventral root is not gender-dependent 188

5.4 DISCUSSION 189

5.5 BIBLIOGRAPHY FOR CHAPTER 5 210

**CHAPTER SIX: GENERAL DISCUSSION 216**

6.1 THE EARLY, PREFERENTIAL LOSS OF MOTOR UNITS PLATEAUS IN THE SYMPTOMATIC PHASE OF DISEASE IN THE $SOD1^{G93A}$ TRANSGENIC MOUSE MODEL OF ALS 216

6.2 DISSOCIATION OF MOTOR UNIT TERRITORY AND FORCE IS DUE TO PREFERENTIAL MOTOR UNIT LOSS 218

6.3 PREFERENTIAL MOTOR UNIT LOSS AND ASSOCIATED REDCTION IN WHOLE MUSCLE FORCE PRECEDES OVERT SYMPTOMS 220

6.4 INCREASING NEUROMUSCULAR ACTIVITY CONFERS PROTECTION TO MOTOR UNITS IN $SOD1^{G93A}$ MICE 221

6.5 EXPERIMENTAL LIMITATIONS 222

6.6 IMPLICATIONS OF ACTIVITY DEPENDENT MOTOR UNIT LOSS IN THE $SOD1^{G93A}$ MOUSE MODEL OF ALS 223

6.7 FUTURE DIRECTIONS 224

6.8 BIBLIOGRAPHY FOR CHAPTER 6 227

## LIST OF TABLES


**CHAPTER 1: INTRODUCTION**

**Table 1-1:** A summary of the most popular motor unit number estimation methods used to study functional loss after injury and disease (Doherty et al., 1995;Daube, 1995;McComas, 1995) 45

**CHAPTER 3: TIME COURSE OF PREFERENTIAL MOTOR UNIT LOSS**

**Table 3-1** Estimates of the number of functional motor units in 3-11 SOD1-G93A mice were made for each of the 4 muscles at 10 day intervals 142

**CHAPTER 4: INFLUENCE OF GENDER ON MOTOR UNIT LOSS**

**Table 4-1** Estimates of the number of motor units in each of 4 muscles were made at 10 day intervals for ~4 to 5 SOD1G93A male (M) and female (F) mice. 160

## LIST OF FIGURES

**CHAPTER 1: INTRODUCTION**


Figure 1-1: The motor unit is made up of the axon of one α-motoneuron originating from the ventral horn of the spinal cord and all the muscle fibers that it innervates. 39

Figure 1-2: Illustration demonstrating the matching between motoneuron and muscle fiber properties. 40

Figure 1-3: Motoneuron die-back results in the denervation of muscle fibers *(A)*, but it can be compensated for by an increase in the activity of surviving motoneurons *(B)* or through compensatory collateral sprouting *(C)*. 41

Figure 1-4: Alternation can cause the overestimation of motor units. 42

Figure 1-5: Motoneuron degeneration in ALS is associated with the recruitment of numerous diverse pathways. 43

Figure 1-6: Anatomical studies have established a clear time course of events associated with the motoneuron degeneration in the $SOD1^{G93A}$ transgenic mouse model of ALS. 44


**CHAPTER 2: PREFERENTIAL MOTOR UNIT LOSS FROM PRE-SYMPTOMATIC $SOD1^{G93A}$ MICE**


Figure 2-1: Schematic illustration of the experimental set up for recording whole muscle and motor unit contractile force from mouse tibialis anterior (TA) or MG muscles. 95

| | | |
|---|---|---|
| Figure 2-2: | Immunohistochemistry for Myosin Heavy Chain Isoforms types I, IIA, not IID/X and IIB in cross-sections of TA muscles. | 96 |
| Figure 2-3: | Changes in the distribution of motor unit forces in the $SOD1^{G93A}$ mouse TA muscles, as compared to age matched $SOD1^{WT}$ control mouse TA muscles. | 97 |
| Figure 2-4: | There was a significant decline in the whole muscle twitch *(A)* and tetanic *(B)* contractile forces in the $SOD1^{G93A}$ mouse TA muscles, and a parallel loss of motor units, as compared to control $SOD1^{WT}$ muscles | 98 |
| Figure 2-5: | Representative cross-sections of 60 day old $SOD1^{WT}$ and $SOD1^{G93A}$ mouse TA muscles | 99 |
| Figure 2-6: | Distribution of fiber cross-sectional area (CSA) in the $SOD1^{WT}$ and $SOD1^{G93A}$ mouse TA muscles. | 100 |
| Figure 2-7: | Changes in the size (*A*) and the number of innervated muscles fibers (*B*) which were classified on the basis of their MHC isoform immunohistochemistry. | 101 |
| Figure 2-8: | Motor unit force declines in MG muscles, in parallel with a decline in whole muscle twitch force and a loss of functional motor units. | 102 |
| Figure 2-9: | A possible mechanism to describe neuromuscular change in pre-symptomatic $SOD1^{G93A}$ mouse TA muscles. | 103 |

**CHAPTER 3: TIME COURSE OF PREFERENTIAL MOTOR UNIT LOSS**

Figure 3-1: A schematic illustrating the procedure of recording of whole muscle and motor unit isometric contractile forces from the hind-limb muscles of mice. 135

Figure 3-2: Comparison of the whole muscle twitch force (*A*) and calculated motor unit numbers (*B*) in the hind-limb muscles of 120- day old non-transgenic wild-type (black bars) and $SOD1^{WT}$ (grey bars) 136

Figure 3-3: The proportion of muscle fibers expressing slow and fast MHC isoforms in the hind-limb muscles of 80-day old $SOD1^{WT}$ mice. 137

Figure 3-4: Whole muscle isometric tetanic forces and the number of intact motor units in the $SOD1^{G93A}$ mouse EDL and SOL muscles at 80-days of age. 138

Figure 3-5: The number of intact motor units in pre-symptomatic 40-day old $SOD1^{G93A}$ and age-matched control (CON) mice. 139

Figure 3-6: The time course of motor unit loss in the $SOD1^{G93A}$ mice, from 40- to 120-days of age. 140

Figure 3-7: The whole muscle isometric tetanic forces produced by the MG and TA muscles in $SOD1^{G93A}$ as compared to control mice. 141

**CHAPTER 4: INFLUENCE OF GENDER ON MOTOR UNIT LOSS**

Figure 4-1: The number of intact motor units in the TA (*A*), MG (*B*), EDL (*C*) and SOL (*D*) muscles of male and female $SOD1^{G93A}$ mice. 158

**CHAPTER 4: THE EFFECTS OF GENDER ON PROGRESSIVE MOTOR UNIT LOSS IN A TRANSGENIC MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS (ALS)** **149**

4.1 INTRODUCTION 149

4.2 METHODS

4.2.1 Generation of $SOD1^{G93A}$ mice 150

4.2.2 Electrophysiological recordings 150

4.2.3 Statistical analysis 151

4.3 RESULTS

4.3.1 Motor unit number 152

4.3.2 Muscle contractile force 153

4.4 DISCUSSION 155

4.5 BIBLIOGRAPHY FOR CHAPTER 4 161

**CHAPTER 5: FUNCTIONAL OVER-LOAD SAVES MOTOR UNITS IN THE SOD1-G93A TRANSGENIC MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS (ALS)** **165**

5.1 INTRODUCTION 165

5.2 METHODS

5.2.1 Generation of $SOD1^{G93A}$ mice 168

5.2.2 Voluntary wheel running 168

5.2.3 Partial denervation of hindlimb muscles 169

5.2.4 Electrophysiological recordings 170

5.2.5 Statistical analysis 171

5.3 RESULTS

5.3.1 Preferential motor unit loss in $SOD1^{G93A}$ mice around the time of symptom onset 172

5.3.2 Voluntary wheel running has gender dependent effects 173

5.3.3 Wheel running accelerates the loss of motor units in TA muscles of male $SOD1^{G93A}$ mice 174

5.3.4 Wheel running saves motor units in the MG muscles of female $SOD1^{G93A}$ mice 175

5.3.5 The effects of voluntary wheel running depend on gender in $SOD1^{G93A}$ mice 177

5.3.6 Partial denervation reduces the number of functional motor units in the TA muscle 178

5.3.7 Partial denervation is also compensated for in the other hindlimb muscles of WT control mice 180
5.3.7 Whole muscle twitch force is maintained in the SOD1$^{G93A}$ mice following avulsion of L4 ventral root 181
5.3.8 Motor units do not become more forceful to compensate for partial denervation in the SOD1$^{G93A}$ mice 182
5.3.9 Avulsion of L4 ventral root confers protection upon the remaining motor untis in the SOD1$^{G93A}$ male mice 184
5.3.10 Motor units are also saved after avulsion of L5 root 186
5.3.11 Protection of motor units following avulsion of L4 ventral root is not gender-dependent 188
5.4 DISCUSSION 189
5.5 BIBLIOGRAPHY FOR CHAPTER 5 210

**CHAPTER SIX: GENERAL DISCUSSION 216**

6.1 THE EARLY, PREFERENTIAL LOSS OF MOTOR UNITS PLATEAUS IN THE SYMPTOMATIC PHASE OF DISEASE IN THE SOD1$^{G93A}$ TRANSGENIC MOUSE MODEL OF ALS 216
6.2 DISSOCIATION OF MOTOR UNIT TERRITORY AND FORCE IS DUE TO PREFERENTIAL MOTOR UNIT LOSS 218
6.3 PREFERENTIAL MOTOR UNIT LOSS AND ASSOCIATED REDCTION IN WHOLE MUSCLE FORCE PRECEDES OVERT SYMPTOMS 220
6.4 INCREASING NEUROMUSCULAR ACTIVITY CONFERS PROTECTION TO MOTOR UNITS IN SOD1$^{G93A}$ MICE 221
6.5 EXPERIMENTAL LIMITATIONS 222
6.6 IMPLICATIONS OF ACTIVITY DEPENDENT MOTOR UNIT LOSS IN THE SOD1$^{G93A}$ MOUSE MODEL OF ALS 223
6.7 FUTURE DIRECTIONS 224
6.8 BIBLIOGRAPHY FOR CHAPTER 6 227

## LIST OF ABBREVIATIONS

| | |
|---|---|
| **4-AP** | 4-aminopyridine |
| **α** | alpha |
| **γ** | gamma |
| **ABC** | avidin-biotin complex |
| **ACh** | Acetylcholine |
| **AChE** | Acetylcholine esterase |
| **mATPase** | myosin ATPase |
| **AHP** | Afterhyperpolarization |
| **ALS** | Amyotrophic Lateral Sclerosis |
| **ALS-PDC** | ALS-Parkinsoniosism-Dementia Complex |
| **AV** | Avulsed |
| **BS** | blocking solution |
| **CLFS** | chronic low frequency stimulation |
| **CMAP** | compound muscle action potential |
| **CON** | control |
| **CSA** | cross sectional area |
| **CV** | conduction velocity |
| **EAAT** | excitatory amino acid transporter |
| **EDL** | extensor digitorum longus |
| **EMG** | electromyography |
| **EPP** | endplate potential |
| **EPSP** | excitatory post-synaptic potential |
| **fMRI** | functional magnetic resonance imaging |
| **FF** | fast fatigable |
| **FI** | fatigue intermediate |
| **GluR-2** | glutamate receptor subunit 2 |
| **HCSMA** | hereditary canine spinal muscular atrophy |
| **HRP** | horseradish peroxidase |
| **IP** | intra-peritoneal |
| **IR** | innervation ratio |
| **MG** | medial gastrocnemius |
| **MHC** | myosin heavy chain |
| **MLS** | myosin light chain |
| **MU** | motor unit |
| **MUAP** | motor unit action potential |

| | |
|---|---|
| **MUNE** | motor unit number estimation |
| **NCAM** | neural cell adhesion molecule |
| **PCR** | polymerase chain reaction |
| **PBS** | phosphate buffered saline |
| **PBS-T** | PBS with 0.1% Tween-20 |
| **PPS** | post-polio syndrome |
| **Rh** | rheobase |
| **Rm** | resting membrane potential |
| **S** | slow |
| **SE** | standard error |
| **SF** | specific force |
| **SOD1** | Cu/Zn superoxide dismutase |
| **SOD1$^{G93A}$** | SOD1 gene with a glycine to alanine substituation at the 93$^{rd}$ codon |
| **SOL** | soleus |
| **TMS** | transcranial magnetic stimulation |
| **TA** | tibialis anterior |
| **V** | voltage |

## Chapter 1

# INTRODUCTION:
# LIMITED FUNCTIONAL COMPENSATION OF SURVIVING MOTOR UNITS IN AMYOTROPHIC LATERAL SCLEROSIS

The disease of Amyotrophic Lateral Sclerosis (ALS) was first described by the French neurologist Jean Martin Charcot in 1869 (Cleveland and Rothstein, 2001). In North America it is also known as Lou Gehrig Disease, after the late American baseball player who contracted the neurodegenerative disease during his career as a New York Yankee. ALS is an adult onset disease that is characterized by the progressive loss of motoneurons from the motor cortex and from the ventral horn of the spinal cord, with resulting weakness in the affected skeletal musculature. Patients with ALS typically have a rapid disease progression with a life expectancy of only 1-5 years from the time of diagnosis. Some forms of ALS, including one conferred by a genetic mutation found commonly in Scandinavian families (Jonsson et al., 2002), and the form which affects the renowned astrophysicist Stephen Hawking have relatively longer progressions (Cleveland and Rothstein, 2001). At present, the prognosis for all ALS patients is poor, as there is no known therapeutic agent available to halt the death of motoneurons. However, there is renewed hope with the recent development of animal models of ALS which allow the screening of potential therapeutic agents as well as characterization of the cellular mechanisms underlying the disease progression.

Management of ALS is complicated by the difficulty in making a definitive diagnosis of the disease. As discussed below, a lack of biological markers for the disease, coupled with trouble associated with ascertaining the presence of both upper and lower motoneuron lesions often delays the diagnosis. Furthermore, the ongoing lower motoneuron loss occurs coincident with compensatory changes in the remaining motoneurons and the skeletal muscles that they innervate which may mask early symptoms of the disease. How effective these compensatory neuromuscular changes are in preventing functional muscle weakness and atrophy is a contentious issue. Characterizing the functional compensation that occurs simultaneously with severe motor unit loss in ALS may help to expedite the diagnostic process of ALS, as well as to devise appropriate therapies to promote the recovery of muscle strength and function of ALS patients through rehabilitation. The **purpose** of the present PhD thesis is to determine the time course of functional motor unit loss and the coincident putative motor unit compensatory processes in a transgenic mouse model of ALS. We also aim to determine strategies that may attenuate the progressive motor unit loss.

## 1.1 MOTOR UNITS: BUILDING BLOCKS OF THE NEUROMUSCULAR SYSTEM

### 1.1.1 Anatomy And Physiology of the Motor unit

The motor unit is comprised of the motor axon of the lower motoneuron and all the muscle fibers that it innervates. The lower motoneurons have cell

bodies in the ventral horn of the spinal cord, and they can be classified into two types based on soma size into the larger alpha (α) and the smaller gamma (γ) motoneurons. The γ-motoneurons innervate intrafusal muscle fibers and regulate length and tension, while the α-motoneurons innervate the extrafusal muscle fibers (Ishihara et al., 2003). In this thesis "motoneuron" refers only to α-motoneurons.

When the summation of the convergent inputs that include the upper motoneurons (originating from the motor cortex), sensory fibers, interneurons and other lower motoneurons causes the motoneuron to depolarize to its threshold for firing an excitatory post synaptic potential (EPSP) is generated. At the axon hillock the EPSP causes the generation of action potential (Burke, 1981), which travels down the peripheral nerve through saltatory conduction (Huxley and Stampfli, 1949). The axon branches before it reaches the target muscle so that it may innervate anywhere from 10 to 10,000 muscle fibers, all of which synchronously contract upon the action potential reaching the neuromuscular junction and causing muscle fiber depolarization (Burke, 1981).

At the neuromuscular junction, the action potential causes the release of acetylcholine (ACh) from the motor axon into the synaptic cleft (Katz and Thesleff, 1957). The ACh dissipates across the cleft and generates the muscle endplate potential (EPP) by binding to ACh receptor gated sodium channels that open to depolarize the muscle membrane (Burke, 1981). The depolarizing current associated with the EPP then spreads down into the junctional folds of the muscle where voltage gated ion channels generate the muscle action

potential that results in the contraction of fibers. The presence of acetylcholine esterase (AChE), which hydrolyzes and thus inactivates ACh, in the synaptic cleft causes the spatial and temporal localization of this nerve-muscle excitation (Katz and Miledi, 1973). However, due to the saltatory conduction the action potential reaches all the axon terminals of one motoneuron and reliably recruits all the innervated muscle fibers of all the motor units. If all the motor units in any one muscle were reliably, synchronously recruited like the collective muscle fibers in a motor unit, then the neuromuscular system would only be able to grade force by recruiting different muscles. Instead, more precise control over contraction in each muscle is achieved by variably recruiting the motor units (Burke, 1981).

### 1.1.2 Intrinsic motoneuron properties and the Size Principle

Motor units are recruited according to a "size principle" that allows the neuromuscular system to produce smooth gradations of force. Differences in the discharge rates of motor units were first noted by Denny-Brown and colleagues (Denny-Brown, 1929). Later, it was shown that in decerebrate cats sciatic nerve stimulation evoked reflex motor unit action potentials that were recorded from the ventral root reflexes and which progressively increased in size in parallel with the amplitude of the stimulation (Henneman, 1957). Henneman later refined his experiments and clearly demonstrated the importance of cell size in determining the probability of a motoneuron to fire in response to a given stimulus (Henneman et al., 1965). According to the Henneman Size Principle the smallest motoneurons are the most likely to be recruited first to a given stimuli

(Henneman, 1985;Burke, 1981). The intrinsic properties that make smaller motoneurons more likely to fire have been extensively investigated and elucidated by the work of Eccles, Kernell, Henneman and Burke.

Motoneuron intrinsic properties that vary in proportion to size include the input resistance ($R_{in}$), rheobase (Rh), duration of the afterhyperpolarization (AHP), frequency of firing and conduction velocity (CV). Slower CV's are found in motoneurons with smaller axon calibers and dendrite trunks and with higher Rm (Kernell, 1966). Therefore, these smaller motoneurons which are the first to be recruited according to the Henneman Size Principle are the most excitable because of high input resistance, but they conduct action potential slower and fire at lower rates than the larger motoneurons (Burke, 1981). The length of the AHP is also well correlated to the CV; motoneurons with longer AHP's tend to have slower CV's and are found to fire in a tonic, rather than phasic fashion (Eccles et al., 1957). The duration of the AHP determines how long a motoneuron remains in a refractory state after an action potential, and therefore it determines the maximum frequency with which a motoneuron can fire. The duration of the AHP of different motor units varies widely, dependent upon the muscle properties that it innervates. Motoneurons that innervate postural, slow-twitch muscles have longer AHP's than the motoneurons which innervate the fast-twitch muscles (Eccles et al., 1957).

Later work established that the rheobase current needed to excite the motoneurons increases with soma size (Gardiner et al., 2006). Also, the threshold of excitation of the next, small motoneuron was reached when the

motoneurons were still firing with their primary range of frequencies (Bakels and Kernell, 1994). In comparison, the magnitude by which large motoneurons must raise their firing frequencies is much greater. Therefore, at lower inputs there is more likely to be an increased recruitment of motor units, rather than an increase in the firing rate of the already recruited motoneurons (Bakels and Kernell, 1994). The intrinsic properties of the small motoneurons allow them to fire slowly, for long periods of time such as those necessitated for postural tasks. The muscle fibers that are innervated by these motoneurons are similarly suited for tonic contractions.

### 1.1.3 The muscle fibers of the motor unit

Muscle fibers can be classified in a number of different ways. Using histochemical methods fibers can be identified on the basis of the sensitivity of their myosin ATPase (mATPase) enzymes to differing pH levels. Three different muscle fiber types can be identified by this mATPase method, slow, intermediate and fast in humans (Brooke and Kaiser, 1970a;Brooke and Kaiser, 1970b). A more sensitive and popular method identifies muscles based on the contractile proteins that they express, such as myosin heavy chains (MHC) isoforms (Pette and Staron, 2000). One single muscle fiber can express multiple isoforms of MHC's as well as myosin light chains (MLC), but usually one type is more dominantly expressed (Staron and Pette, 1987). Using immunohistochemistry, the predominant MHC isoforms can be detected in a muscle. This method has the advantage of being able to detect four different types of muscle fibers, type I,

IIA, IID/X and IIB as well as the co-expression of more than one MHC isoform (Hämäläinen and Pette, 1993).

The type I fibers are the smallest in diameter and are capable of producing the least force of any of the fibers in mammalian muscles (Bottinelli, 2001). They are usually found in high abundance in postural muscles, as well as other muscles that have to be active for large portions of the day. Usually, muscles with a high proportion of type I fibers appear "red" because these muscles are oxidative, and thus have dense capillary beds and are rich in myoglobin and mitochondira (Burke, 1981). Type I fibers are most abundant in humans and other large mammals, and as the size of the mammal decreases, so does the proportion of type I fibers. For example, the anti-gravity soleus muscle is composed of only type I fibers in the cat, but in the mouse less than half the soleus fibers are type I (Krutki et al., 2006;Hämäläinen and Pette, 1993;Wang and Kernell, 2001). It has been postulated that the relatively low proportions of type I fibers in smaller mammals is correlated with a decrease in the amount of weight borne by the muscles, indicating that the loading of muscles determines, at least in part, their muscle fiber compositions (Wang and Kernell, 2001). In all of the mammalian hindlimb muscles that have been studied, the type I fibers have been found to be localized to the deeper regions, while paler, more forceful fast-twitch muscles fibers are found in the more superficial areas (Fig 1-1). The localization of the muscle fibers may be related to the need to preserve heat, or to protect the more vulnerable type I fibers from damage (Wang and Kernell, 2001).

Type II muscle fibers are bigger and more forceful than type I fibers (Bottinelli, 2001). The largest, most forceful fibers are type IIB and IID/X. These fibers appear pale because they rely on glycolysis for most of their energy demands and therefore do not require as dense capillary beds as the smaller, type I fibers. Also, the myoglobulin and mitochondrial content of type IIB and IID/X fibers are relatively lower than the type I fibers, further contributing to their pale appearance. Type IIA fibers are intermediary, and rely on both oxidative and glycolytic pathways to produce energy (Kernell et al., 1995). The type IIB and IID/X muscle fibers are found in motor units that tend tend to be active for only short periods of time throughout the day, as compared to the postural "red" muscles (Hensbergen and Kernell, 1997). The size, forcefulness and fatigability of muscle fibers fibers is in the order of I<IIA<IID/X<IIB. Type IIB fibers do not exist in human muscles (Pette and Staron, 2000).

Different muscles contain different proportions of muscle fiber types depending upon their function and their daily activity patterns. However, all the muscle fibers in a motor unit are homogenous with respect to their metabolic enzyme activities (Nemeth et al., 1981) and to their contractile properties (Burke, 1981). The contractile properties of the muscle unit are well matched to the properties of the motoneurons that they are innervated by (summarized in Fig 1-2; Bakels and Kernell, 1993c). For example the small motoneurons with the longer duration AHP's, which are recruited first and can fire in a tonic fashion, innervate type I muscle fibers (Fig 1-2*A*). These fibers generate less force per area, but are capable of contracting for longer periods of time, such as during

postural tasks when the motoneurons would be activated (Burke, 1981). The small motor units have less fibers per motoneuron (innervation ratio). These small motoneurons and fatigue resistant muscle fibers comprise the slow (S) motor units (Bakels and Kernell, 1993b). In faster muscles, the relationship between the fast fatigable (FF) and fast intermediate (FI) motor units and the intrinsic motoneuron characteristics is not as clear as in the slower muscles, but on average these properties do correspond (Bakels and Kernell, 1993a). Nonethelesss the force is directly related to both the cross sectional area and the IR (Fig 1-2*B*.C; Tötösy de Zepetnek et al., 1992).

### 1.1.4 Changing motor unit activity causes adaptations in both motoneuron and muscle fiber properties

The studies of Kernell have established that motor unit properties are matched to the discharge properties of the motoneuron. The importance of dynamic regulation of muscle properties was first described in the 1930's and 1940's when electrical stimulation was found to prevent disuse atrophy of muscle after tenotomy or spinal cord injury (Eccles, 1944) and maintain muscle mass and some strength (Fischer, 1939). These studies were extended to show that when slow- to fast- twitch muscles were cross-reinnervated they took on the properties associated with the new nerve (Buller et al., 1960). This change in contractile properties of muscle fibers is related to the activity patterns imposed by the cross-reinnervating nerve. In the absence of neuromuscular activity, following a spinal cord lesion or tenotomy, the contraction time of the slow-twitch

soleus muscle reduces (Vrbova, 1963). This change in muscle contractile properties is prevented by imposing a low frequency pattern of stimulation upon the nerve innervating the soleus muscle. Only slow frequency stimulation, which approximates the frequency that the soleus muscle would normally be stimulated with during daily activity maintains the muscle properties (Salmons and Vrbova, 1969). Since this time the effects of chronic low frequency electrical stimulation (CLFS) on motoneurons and muscles have been widely studied.

When CLFS is imposed upon motor units, there is a conversion of muscle fibers to slower phenotypes within 14 days of continuous stimulation in rats (Delp and Pette, 1994;Putman et al., 2001). Following ~3 month of stimulation of the cat medial gastrocnemius nerve (MG) the muscle fibers all convert to type I phenotypes (Gordon et al., 1997). In association with this conversion the excitability of the motoneurosn increases due to a decrease in the rheobase and an elevation of the resting membrane potential. The shift to slower motoneuron phenotypes is further evidenced by a slowing of the conduction velocity and an increase in the duration of the AHP (Munson et al., 1997).

Neuromuscular activity can also be increased through exercise paradigms. When laboratory rodents are given access to running wheels they become very active; female mice have been shown to volitionally run ~15km each night in short explosive bouts, while the distance run by male mice is ~40% less (De Bono et al., 2006). Such voluntary running activity has been shown to change the proportion of fast-twitch muscle fibers in rats, causing a decrease in the proportion of the most forceful fast-glycolytic fibers (Ishihara et al., 1991;Gallo

et al., 2006). The biophysical properties of the motoneurons innervating these fibers also change, but only in the smaller motoneurons that could be identified by a AHP of more than 20 ms (Gardiner, 2006). Following 12 weeks of free access to rodent wheels these smaller motoneurons became less excitable due to hyperpolarization of the resting membrane potential, an increased rheobase and a significantly greater AHP amplitude (Beaumont and Gardiner, 2002).

There were no changes reported in any of the properties of the larger motoneurons following voluntary exercise, because they presumably would only be activated during more intense sessions of activity. Changes in the large motoneurons were indeed seen after rats were forced to partake in high intensity training for ~2 hours per day. After this "endurance training" all motoneurons, regardless of size became less excitable (Beaumont and Gardiner, 2003). Motoneuron and muscle fiber properties are differentially altered by voluntarily exercise, with muscle fibers converting to slower phenotypes and motoneurons changing to resemble the motoneurons that innervate fast-twitch muscle fibers (Beaumont and Gardiner, 2003). On this basis it has been postulated that the reduced excitability of the motoneurons may be an adaptation to the increase in excitation from descending pathways, including seretonergic projections (Gardiner et al., 2006). The activity of seretonergic projections into the spinal cord are known to increase under certain conditions, including locomotor activities such as treadmill running (Jacobs et al., 2002). According to this hypothesis, motoneurons could only become excitable if there was no increase in seretonergic projections, or other excitatory pathways coincident with the

increased neuromuscular activity. In agreement with this hypothesis, the only time motoneurons were reported to increase their excitability following a period of elevated motor unit activity was following chronic nerve stimulation (Munson et al., 1997). As the cats in this experiment did not change their daily levels of activity, it can be postulated that their descending excitatory pathways remained unaffected. Therefore, motoneurons did not have to adapt to increasing excitability, and could convert to slower, more readily recruitable phenotypes associated with S motor units. However, the mechanisms that regulate changes in motoneuron intrinsic properties have yet to be elucidated.

It is becoming increasingly clear that motor unit properties do not rely solely on the activation pattern of lower motoneurons. Other factors, including the descending pathways from the motor cortex may also influence the intrinsic properties of the motoneuron, and by extension the recruitment pattern of motor units. Neurotrophins released from the muscles and the nerves may also influence motor unit properties, further complicating the determination of motor unit phenotypes. Over the past few years Edgerton and his group have demonstrated that the neural control over muscle properties is incomplete. When motor unit activity was reduced by both spinal cord isolation and nerve crush, the two conditions were found to have additive effects in promoting conversion to fast muscle fiber types. These effects suggest that motoneurons which were inactive, but still retain their nerve muscle connections were able to influence the contractile properties of muscle fibers (Roy et al., 1996). More recently, this group shows that the proportions of the different type II MHC

isoforms in fast-twitch rat hindlimb muscles did not differ according to daily, voluntary activity (Hodgson et al., 2005).

The cellular mechanisms that underlie these putative changes in muscle fiber contractile properties independent of neuronal activity remain to be elucidated. Such mechanisms have the potential to complicate our interpretation of changes in motor unit properties in diseases where systems may be affected. In ALS, where both upper and lower motoneurons are lost, the adaptive changes of the motor unit will most likely be influenced by changes in descending pathways as well as in the ventral horn of the spinal cord.

### 1.1.5 Partial denervation and motor unit adaptations

The studies reviewed above concern adaptive changes of the motor unit in response to variations in the activity levels of normal neuromuscular systems. Studies that better model the motor unit responses during ongoing degeneration in ALS involve the partial denervation of muscles. In these studies, a proportion of the functional motor units in a particular muscle or group of muscles have been experimentally removed, and changes in the remaining motor unit properties have been observed.

A loss of motor units due to injury or disease may be compensated in one of two ways (Fig 1-3). As the number of motor units innervating a specific muscle decreases, the recruitment of the remaining motor units increases in parallel (Schulte-Mattler et al., 2000). This may be mediated by a similar mechanism as the one which causes a compensatory increase in the firing

pattern of motor units in association with early adaptations to strength training (Patten et al., 2001). When the time course of recovery from partial denervation was tracked in rats using electromyographic (EMG) recordings, it was found that initially the activity of the remaining motor units increased over the first week of denervation. As motor unit enlargement occurred at ~12 days, the activity of the motor units gradually decreased (Slawinska et al., 1998).

It has long been known that partially denervating mammalian skeletal muscle provokes a compensatory process whereby the remaining motor units undergo axonal sprouting and reinnervate denervated muscle fibers (Brown et al., 1981). This results in the enlargement of remaining motor units, and a consequent increase in their forcefulness. The magnitude of enlargement is the same for all motor units; motor units can enlarge up to 5 times in the mouse and up to 8 times in the cat (Brown and Ironton, 1978;Gordon et al., 1993). Axonal sprouting can be intranodal (emanating from between the nodes of the axons) or ultraterminal (from the terminals of the axons; Brown et al., 1980). These sprouts are guided to denervated endplates by the terminal Schwann cells which form bridges (Son and Thompson, 1995). The reinnervated muscle fibers transform to take the on the characteristics of the other muscle fibers in the motor unit (Fig 1-3).

Although axonal sprouting can effectively compensate for the loss of intact motor units, even a healthy neuromuscular system cannot support prolonged periods of motor axonal sprouting or enlarged motor units indefinitely. When prolonged compensatory axonal sprouting was provoked by repeated neuronal

injuries in the rat (Harding et al., 1998), or by the over-expression of growth associated proteins in transgenic mice (Harding et al., 1999), the remaining motor units became increasingly susceptible to cell death. The danger of prolonged axonal sprouting may be especially relevant in ALS, where neurodegeneration continues throughout disease progression (Cleveland and Rothstein, 2001).

When motor unit loss is not ongoing, enlarged motor units can be maintained for periods of up to 20 years, after which their increased activity causes them to become vulnerable to degeneration (Rodriquez et al., 1995). This phenomenon is likely to be responsible for post polio syndrome (PPS), in which patients who recovered from polio begin to experience later onset paralysis (McComas et al., 1993). In patients where motor units do enlarge it has been estimated that increase in the motor unit forcefulness is able to compensate for the loss of up to 90% of the motor units (McComas et al., 1971). This enlargement can be measured using EMG or force measurements, with the two corresponding well in healthy systems. However, the reinnervation, and the consequent conversion of muscle fibers causes muscle fibers with homogenous properties to "clump" together (Rafuse and Gordon, 1996). This clumping may confound studies with needle EMG which relies on measures of muscle fiber density to estimate the size of motor units in the vicinity of the recording needle. For this reason, care must be taken when interpreting results obtained from needle-EMG studies in ALS patients, as well as in the animal model of ALS.

### 1.1.6 Motor unit number estimations

The enumeration of motor units becomes necessary when determining the extent of a lower motoneuron lesion due to progressive disease or injury or assessing the efficacy of potential therapeutic agents. Motor unit characterization can also be useful in assessing the potential of rehabilitation strategies that may promote functional recovery. Over the past 40 years a number of methods have been developed for motor unit number estimation (MUNE; Doherty et al., 1995). The main assumption of all MUNE methods is that the recruitment of a single motor unit will result in the all-or-none incremental increase in the force or electrical activity produced by the muscle (Doherty et al., 1995). Measuring the fluctuations in the either the whole muscle force or electrical activity will therefore give a measure of the forcefulness or the size of the motor unit action potential (MUAP) of a motor unit. These fluctuations can be provoked either by applying a graded stimulus to a nerve or by measuring incidental fluctuations during voluntary movement through a variety of methods that are reviewed by Doherty et al., (1995) and Daube (1995). The methods described in these reviews are also summarized in Table 1-2.

The incremental method of MUNE was developed by McComas in 1967 (McComas, 1995) and involved the detection of an M-wave associated with a single motor unit in response to threshold stimulation (the motor unit action potential; MUAP). The maximum M-wave of the muscle was then divided by MUAP estimate the number of motor units in the muscle. Later, the technique

evolved because the number of sampled motor units had to be increased in order to ensure a representative sample. More motor units were recruited by gradually increasing the stimulus intensity. The resulting all-or-none increments in the M-wave were recorded, and each step-wise increase was assumed to be due to the addition of a motor unit (McComas et al., 1971). The main assumption of this incremental MUNE method is that the motor units summate in a reproducible and predictable order. However, this is not always the case, and motor units with similar thresholds may be recruited in several combinations. This variable recruitment is called "alternation" and may result in the overestimation of the motor unit number (Fig 1-4). As the number of estimated motor units in a muscle increases, so does the error associated with alternation (McComas, 1995).

In human subjects it is difficult to estimate the effect of alternation because there is no true "gold-standard" for motor unit numbers in any mammalian muscle (Enoka, 1995). Anatomical estimates of motor unit numbers in particular limbs are difficult to obtain because of the presence of sensory neurons. Although these sensory neurons tend to have a smaller diameter, a distinct double peaked distribution is not always seen in histograms of axon calibers (Enoka, 1995;Thangam et al., 1989). Even if the proportions of α-motoneurons in a muscle are determined, these proportions are highly variable, and such estimates are prone to experimental error (Enoka, 1995). In recent years, many methods of MUNE have tried to overcome the problem of alternation by not relying on incremental increases in force or MUAPs in order to estimate motor unit sizes. For example, in the multiple point stimulation method of estimating

MUNE the experimenter stimulates the axon with the lowest threshold at one particular site of the motor nerve (Doherty et al., 1995). Once the MUAP of this motor unit is recorded the stimulating electrode is moved to another site, where a different motor unit is assumed to have the lowest threshold (Doherty et al., 1995). This method requires access to a long stretch of the motor axon. A quick review of table 1 reveals that none of the MUNE methods currently used in human subjects are completely free of errors or technical complications. Some of these errors have been addressed in animal models with various degrees of success.

The incremental method has been adapted for use in experimental animals such as rats (Fu and Gordon, 1995a;Fu and Gordon, 1995b) cats (Rafuse et al., 1997) and mice (Chapters 2-5). The problem of alternation was addressed in these experiments by reducing the number of motor units that can summate at any one time. This reduction was done by dissecting the ventral root, and teasing the root out into small filaments. Each of these filaments encased only a small proportion of the motor axons that innervated the muscle. Hence, when the filaments were stimulated according to the incremental method of MUNE, only a small number of all-or-none steps in the whole muscle force were detected (Fu and Gordon, 1995b). By changing the filament that was stimulated different motor units could be recruited to increase the sample size and reduce the error associated with an unrepresentative sample. Although this method has been used with great success in larger experimental animals, it is not well tolerated by smaller rodents including mice because of the laminectomy required to tease the

ventral roots. In the present thesis, a novel technique is described (Chapter 3) which attempts to reduce the alternation associated with the incremental method used in mice.

## 1.2 COMPENSATORY MOTOR UNIT CHANGES IN ALS PATIENTS

### 1.2.1 Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is the most common motoneuron disorder, with an incidence of approximately 1:100,000. Approximately 90% of all ALS cases are considered idiopathic, and at present only a few, controversial risk factors are associated with ALS (Armon, 2003). In sporadic ALS, diagnosis of disease is usually made in the fifth or sixth decade of life, with a significantly earlier age of onset in men (54.3 years of age) than in women (61.5 years of age (Rudnicki, 1999). Gender also influences the incidence of ALS, and the ratio of male and female patients is ~1.6 to 1 at the mean age of onset (Cashman et al., 2000). The male to female ratio is highly variable depending on the sample size and location of the cohort under study (Militello et al., 2002;Magnus et al., 2002;Rudnicki, 1999). The mean survival time from the age of diagnosis is ~2 to 5 years with an average survival of 47 months in both genders (Magnus et al., 2002). The initial clinical presentation of sporadic ALS is most commonly in a distal limb. A rarer form of ALS presents with bulbar symptoms, including fasiculations in the tongue, dysarthria and dysphagia. The bulbar form of ALS is more common in women and in patients over the age of 80 (Traynor et al., 2000).

One of the most important risk factors of developing ALS is a history of disease in the family. Approximately 10% of all ALS cases are considered to be associated with inherited genetic mutations, and in the past decade several of these mutations have been mapped to specific loci (Majoor-Krakauer et al., 2003). The first genetic mutation to be identified was a mutation in the ubiquitous antioxidant gene, superoxide dismutase (SOD1) (Rosen et al., 1993). SOD1 is an antioxidant enzyme is responsible for the conversion superoxide to hydrogen peroxide (Maier and Chan, 2002). The most commonly studied SOD1 mutation involves a glycine to alanine substitution at the 93$^{rd}$ codon (SOD1$^{G93A}$) (Rosen et al., 1993). This mutation is thought to confer a yet to be determined gain of cytotoxic function upon the SOD protein, but it does not appear to interfere with its antioxidant capacity (Rabizadeh et al., 1995). Recent work using transgenic mice that express the SOD1$^{G93A}$ gene has increased our knowledge of the pathophysiology of ALS (Cleveland and Rothstein, 2001).

Other genetic mutations have also been recently linked to yet to be identified genetic loci, and are currently designated as genes ALS2-5 (reviewed by (Majoor-Krakauer et al., 2003). The identification of carriers of mutations associated with ALS has allowed researchers for the first time to track presymptomatic changes in motor unit numbers (Aggarwal and Nicholson, 2002). Characterizing these early changes in the motor units of pre-symptomatic ALS patients may help expedite the diagnosis of ALS. When treatments do become available for motoneuron degeneration, the early diagnosis of functional motor unit loss will be critical for the effective reversal of symptoms.

### 1.2.2 Diagnosis of ALS

Motoneuron loss initially presents as weakness in the affected limbs. Careful review of medical records reveals that many patients report that the affected limbs have been weak for many years prior to diagnosis (Swash and Ingram, 1988). This early weakness may be compensated for by above mentioned adaptations in motor unit properties, and by adaptive strategies including co-contraction of agonist muscles (Bromberg and Larson, 1996). However, weakness is a common complaint in many other disorders, which must be excluded before a diagnosis of ALS can be obtained (Preston and Shapiro, 1998). The main EMG findings that characterize ALS are ongoing reinnervation/denervation as seen by changes in the size of the motor unit amplitude. Moreover, for a diagnosis of ALS, signs of denervation must be found in at least three of the four body segments (cervical, thoracic, lumbar and sacral) and often, denervation is found in limbs in which the patient has no prior complaint of weakness (Preston and Shapiro., 1998). The necessary involvement of upper motoneuron lesions is difficult to diagnose because upper motoneuron signs can often be masked by lesions in lower motoneurons (Kaufmann et al., 2004). Fortunately, upper motoneuron lesions can now be better diagnosed with transcranial magnetic stimulation (Osei-Lah and Mills, 2004;Pohl et al., 2001)

### 1.2.3 Prognosis and Treatment of ALS

At present the prognosis for ALS patients is poor as there are no pharmaceutical agents known to halt or slow the degeneration of motoneurons. The life-expectancy of ALS patients varies greatly, depending on several factors, including age at diagnosis, site of disease onset and gender. Younger patients tend to survive for longer, as do patients with limb onset ALS as compared to bulbar onset and females compared with males (Magnus et al., 2002). Some familial forms of the disease are also associated with a better prognosis than others. Patients with the D90A-SOD1 mutation have an average survival of ~19 years (Jonsson et al., 2002). However, most known familial mutations are associated with a very rapid disease progression.

Currently, only the antiglutaminergic drug, riluzole is approved for therapeutic use in ALS patients in North America. Riluzole has a modest effect on survival, ranging between 2 to 6 months (Bensimon et al., 1994), but other anti-glutaminergic drugs do not have similar effects (reviewed by Rowland and Shneider, 2001). Unfortunately, antioxidants, anti-inflammatory drugs, neurotrophic agents and anti-viral drugs have also not affected disease progression. More recently, clinical trials of creatine that may help maintain muscle mass and prevent mitochondrial dysfunction have also had disappointing results, having failed to affect disease progression (Shefner et al., 2004;Groeneveld et al., 2003). Currently, there is much excitement surrounding treatments that combine different pharamaceutical agents, which have previously not affected disease progression when administered on their own. These drug

"cocktails" have been shown to slow disease progression in mouse models of ALS (Kriz et al., 2003). Other new therapeutic agents that have not yet been tested in clinical trial, but have shown promise in halting disease progression in mouse models, are drugs which induce the production of heat shock proteins (Benn and Brown, Jr., 2004). One possible mechanism of action of heat shock inducing proteins will be discussed below.

### 1.2.4 Pathophysiology of upper motoneuron loss in ALS

Changes in the motor cortex are now being explored with the use of technology including functional magnetic resonance imaging (fMRI) and transcranial magnetic stimulation (TMS). Using TMS, it was revealed that the cortical motoneurons are hyperexcitable probably due to the degeneration of inhibitory interneurons (Zanette et al., 2002a) and a decrease in the resting membrane potential of the surviving motoneurons (Zanette et al., 2002b). The earliest hypothesis regarding degeneration in ALS was proposed by Charcot and suggested that motoneurons "die-forward" with the initial insult originating in the motor cortex, but this has yet to be fully explored (Eisen and Weber, 2001).

Plasticity at the motor cortex level may help to compensate for early initial motor unit loss. Indeed fMRI studies have shown that the brain area activated during locomotor tasks becomes more diffuse in ALS patients (Konrad et al., 2002). This increased recruitment of additional motor areas is not thought be associated with functional reorganization of cortical maps, but rather the use of established cortical pathways that would normally only be recruited during

difficult tasks in healthy patients (Schoenfeld et al., 2005). Whether such compensatory processes can be optimized to aid in the rehabilitation of ALS patients has yet to be determined.

### 1.2.5 Physiology of motor units in ALS

The diagnosis of ALS is complicated by the ongoing reinnervation and denervation seen in the affected skeletal musculature. According to data from different neuropathic ailments, the reinnervation of partially denervated muscles by axonal sprouting should be able to compensate until only ~10% of the motor units remain (McComas et al., 1971;Gordon et al., 1993). The established diagnostic criteria for ALS require that there be widespread denervation evidenced by EMG studies, despite weakness presenting usually in only one distal limb (Preston and Shapiro, 1998). When motoneuron loss and weakness are tracked in pre-symptomatic familial ALS patients, significant loss of functional motor units does indeed precede the presentation of weakness (Aggarwal and Nicholson, 2002). Motor unit loss is not restricted to the limbs, and at the time of symptom onset in the distal limbs, evidence of denervation in the diaphragm can be found in approximately a third of all ALS patients whether they had difficulty breathing or not (Stewart et al., 2001). These findings of asymptomatic motor unit loss from the limbs and diaphragms of ALS patients suggest that there are some compensatory processes that mask early degeneration. Findings that there is a loss of ~50% of the motor units in the affected weakened limbs of ALS patients at diagnosis suggests that the compensatory processes are not as effective as in

other conditions of partial denervation due to injury or neuropathic processes (Hansen and Ballantyne, 1978). The extent to which motor units do enlarge in ALS patients varies widely, depending upon a number of factors, including the age of the patient and the severity of the disease (Carleton and Brown, 1979).

When the motor unit size was estimated using EMG recordings, it was found that the motor units in younger, less severely affected patients tended to enlarge more readily than those in older, more severely affected patients (Dengler et al., 1990;Schmied et al., 1999). The magnitude of these changes was quite small as compared to patients with spinal muscular atrophy (SMA) and post-polio syndrome (PPS), and there were very few signs of motor unit enlargement by collateral axonal sprouting in ALS patients as compared to the other, more slowly progressing conditions (Vogt and Nix, 1997). The longitudinal characterization of motor units using EMG recordings in ALS patients did reveal some remodeling, but only in a small proportion of the units that were studied, and it was suggested that the compensatory capacity of motor units through compensatory collateral sprouing declined with disease progression (Sharma and Miller, 1996). Another longitudinal EMG study of motor unit size revealed that units also decreased their size; it was suggested that this decrease occurred because the disease progresses too quickly for there to be adequate compensation (Carleton and Brown, 1979). Indeed, in the pre-symptomatic familial ALS patients, the motor unit loss progressed very quickly once the number began to drop below normal levels (Aggarwal and Nicholson, 2002).

The modest enlargement of the motor units seen in some of the EMG studies was not paralleled by an equal increase in the forcefulness of the motor units (Milner-Brown et al., 1974;Schmied et al., 1999). This dissociation between the size of the motor unit and the forcefulness of the motor unit may be related to a dysfunction at the neuromuscular junction, which would lead to increased fatigue. However, a recent study showed no abnormalities in the muscle fibers of ALS patients (Krivickas et al., 2002). It has been shown that muscle fatigue brought on by 30 seconds of maximal voluntary isometric contractions may be related to an increase in the proportion of type I muscle fibers (Sanjak et al., 2004). As the specific force of type I fibers is lower than type II fibers in humans (Bottinelli, 2001) a change in the proportions of fibers may be causing the dissociation between the size of the motor units and their forcefulness. However, as muscle fiber biopsies are not usually done in ALS patients, this possibility has not been well explored.

Dissociation of motor unit size and forcefulness is further evidenced by the non-parallel decline in the compound muscle action potential (CMAP) and the motor unit number (Arasaki et al., 2002;Venkatesh et al., 1995;Sharma and Miller, 1996). In a study which differentiated between severely and less severely affected ALS patients, the ratio of motor unit force and CMAP was found to decline in the more severely affected patients, indicating that there was some electromechanical dysfunction which worsened with disease progression (Venkatesh et al., 1995). This electromechanical insufficiency greatly complicates the monitoring of disease progression in ALS patients, as the

correlation between CMAP and pinch power is not very robust (Arasaki et al., 2002). Instead, the estimated number of motor units was found to be a much better indicator of disability (Arasaki et al., 2002) as well as functional strength and survival (Armon and Brandstater, 1999). Therefore, care must be taken when interpreting results from EMG recordings in ALS patients, especially in the evaluation of potential therapeutic drugs.

The advent of the animal model of ALS (Gurney, 1994) now allows the full characterization of motor units during the entire course of disease. Understanding how motor unit properties change throughout the disease process will hopefully further our understanding of the cellular mechanisms underlying motoneuron degeneration and the changes in properties of motor units. Once we are successful in halting or slowing the progressive loss of functional motor units it will be important to devise strategies to increase their forcefulness and thus effectively compensate for losses suffered during the pre-symptomatic phase of disease.

## 1.3 MOTOR UNITS IN ANIMAL ALS MODELS

### 1.3.1 Animal models of ALS

There are a variety of conditions under which mammals exhibit pathology and symptoms that resemble sporadic ALS disease. The Guamian neurological disorder ALS-Parkinsonionism-dementia (ALS-PDC) complex has been linked to consumption of either flour made out of washed cycad nuts or fruit bats who have eaten the cycad nut (Cox and Sacks, 2002). When washed cycad nuts are fed to

healthy mice they develop a pathophysiology and symptoms highly reminiscent of the human disease (Wilson et al., 2002). This model has been used to establish the sensitivity of imaging techniques to detect degeneration in the pre-symptomatic phase of disease (Wilson et al., 2004) but its applicability to study disease process in sporadic ALS is limited. Other forms of ALS can be found in animals with random genetic mutations, including canine and equine forms of ALS-like disease that are caused by unknown familial gene mutations.

Motor units have been well characterized in hereditary canine spinal muscular atrophy (HCSMA), and it has been found that motor axons never mature completely. Beginning at 6-8 weeks, the dogs homozygous for HCSMA develop weakness in their paraspinal muscles, which progresses from caudal to rostral directions (Pinter et al., 1995;Cork et al., 1997). The slow motor units are preferentially affected in this phenotype, with a decreased quanta of neuromuscular transmitter resulting in ineffective transmission and failure of excitation-contraction coupling (Carrasco et al., 2004). Therefore, weakness is apparent prior to motor unit loss, due to the neuromuscular transmission failure, which can be temporarily reversed by the administration of the potassium blocker, 4-aminopyridine (4-AP;Pinter et al., 1997). However, in a clinical trials where a similar agent, 4-diaminopyridine that also enhances the release of neurotransmitters at the neuromuscular synapse was given to ALS patients motor impairment was not prevented (Aisen et al., 1996). This inability to prevent neuromuscular fatigue suggests that failure of transmission is not the key element causing weakness in human ALS patients. Recently, it has been

suggested that due to involvement of the motoneurons innervating medial muscles, HCSMA may be a more accurate representation of SMA, rather than ALS (Carrasco et al., 2004).

Other models of ALS that involve unknown genetic mutations are the wobbler, pmn, wasted and mnd mice, which all have varying neurophysiological impairments (reviewed by Doble and Kennel, 2000). With the advent of transgenic mouse models that more accurately recapitulate the course of disease in human patients the use of these non-transgenic models has declined. The most commonly used transgenic mouse model of ALS was developed by over expressing human mutant SOD1 with a glycine to alanine conversion at the 93$^{rd}$ codon (Gurney, 1994). These mice develop bilateral hind limb weakness approximately 90 days after birth, coincident with a significant loss of motoneurons from the lumbar spinal cord (Gurney et al., 1994;Chiu et al., 1995). The SOD1$^{G93A}$ mice display complete hind limb paralysis by 130 days of age and expire soon thereafter, most likely due to problems breathing (Chiu et al., 1995). Other SOD mutations have now been linked to familial ALS, and as a result there are now other transgenic mouse models available for research including the SOD1$^{G37R}$ and SOD1$^{G85R}$ mice which are currently being characterized. However, the use of transgenic mouse models of ALS has been criticized by some opponents who have postulated that the high copy number of the mutant gene may be responsible for some aspects of the ALS-like disease (Bergemalm et al., 2006). In the absence of reliable non-transgenic mouse models that

accurately recapitulate the human disease phenotype ALS researchers continue to use the popular SOD1 transgenic mouse models.

### 1.3.2 The SOD1$^{G93A}$ mouse model of ALS

The glycine to alanine conversion at the 93$^{rd}$ codon confers a yet to be determined gain of cytotoxic function to the antioxidant SOD. It had been previously hypothesized that the change in the tertiary structure of the SOD as a result of the G93A mutation interfered with SOD's antioxidant capacity and perhaps even caused it to start producing reactive oxygen species (Crow et al., 1997b;Crow et al., 1997a). The exact cytotoxic function that is gained has yet to be determined (Cleveland and Rothstein, 2001). Recent theories about the cytotoxic properties of SOD1$^{G93A}$ have focused on the aberrant folding of the SOD1, which would cause the accumulation of the SOD1 protein (Stathopulos et al., 2003;Deng et al., 2006;Benn and Brown, Jr., 2004). This accumulation is hypothesized to strangle the axon processes (Cleveland and Rothstein, 2001). As motoneurons are some of the longest cells in the body, affecting the axon processes in such a way would have an almost immediate effect, and would result in die-back of the motor axons, with the neuromuscular junction being the first to be affected. The aggregation of misfolded SOD may also be critical since the level of chaperone protein that would normally neutralize the effects of misfolded proteins is decreased in the spinal cord of SOD1$^{G93A}$ mice (Bruening et al., 1999). Increasing the activity of proteosomes through the induction of heat shock proteins has been shown to save motoneurons in the SOD1$^{G93A}$ mouse

model of ALS (Kieran et al., 2004). Treatment with arimoclomal to induce heat shock proteins was effective in prolonging the life of $SOD1^{G93A}$ mice even if the drug was administered after the onset of symptoms (Kieran et al., 2004).

A possible scheme of the different pathways involved in motoneuron die-back in ALS is illustrated in figure 1-5 (adapted from (Cleveland and Rothstein, 2001). A number of different factors interact to cause motoneuron loss from the lumbar spinal cord, including activation of the astrocytes and microglia, changes in glutamate receptors and of course, an increase in free radical formation which cannot be effectively buffered and hence leads to oxidative stress. Activated astrocytes and microglia are known to release free radical species (Cassina et al., 2002)and glial cells cultured from $SOD1^{G93A}$ produce prostaglandins and other pro-inflammatory molecules that might be involved in perpetuating the cycle of inflammation and degeneration in ALS (Hensley et al., 2006). However, a more important factor in ALS may be that the excitatory amino acid transporters (EAAT) may be disrupted on the astrocytes, thereby increasing the amount of glutamate available at the synapse (Rao et al., 2003). Furthermore, in some cases of human sporadic ALS a subset of the glutamate transporter subunits (GluR-2) are not effectively edited before insertion into motoneurons (Kawahara et al., 2004). Motoneurons usually have only a small complement of these channels, and therefore they may have an increased sensitivity to any changes to the GluR2 subunit (Kawahara et al., 2003). The inclusion of unedited GluR2 is associated with an increase in the permeability of glutamate channels to calcium; an influx of calcium to the motoneuron can initiate

neurodegeneration (Das et al., 2005), promote further misfolding of the SOD1 molecule (Tateno et al., 2004) and inactivate the EAAT transporters on the glial cells (Boston-Howes et al., 2006). How these different pathways of motoneuron degeneration are activated in ALS has not yet been determined, but it is becoming increasingly clear that a multi-pronged approach is required to save motoneurons from degeneration.

### 1.3.3 Motoneuron pathology in the SOD1$^{G93A}$ transgenic mouse

Although the onset of overt weakness in the SOD1$^{G93A}$ mouse coincides with the significant loss of motoneurons from the lumbar spinal cord (Chiu et al., 1995) motoneurons start to "die-back" in the presymptomatic stage of disease (Frey et al., 2000) and by symptom onset many of these motoneurons would have lost their connections to muscles. Therefore, it is important to investigate the time course of functional motor unit loss in the SOD1$^{G93A}$ mouse model of ALS.

The first sign of pathology in the SOD1$^{G93A}$ mouse model of ALS is seen in the axons and in hind limb muscles. The mutant SOD1$^{G93A}$ protein accumulates in the motor axons (Johnston et al., 2000) and in the gastrocnemius muscle (Turner et al., 2003) between 30 and 40 days of age, coincident with vacuolar changes in the lumbar spinal cord and the proximal axons (Chiu et al., 1995). Denervation in the muscles is apparent by 50 days of age, when the first signs of axon loss from the ventral roots are also seen (Fischer et al., 2004). At 47 days of age the CMAP is already significantly reduced by 15% and there are

fasiculations in one third of the mice (Kennel et al., 1996). These functional changes occur coincidentally with the dying back of motor axons (Fischer et al., 2004). The loss of motor axons continues and by 120 days the number of somatic motoneurons from the brain stem is significant reduced and cortical white matter begins to undergo "Wallerian degeneration" (Chiu et al., 1995;Dal Canto and Gurney, 1995). Upper motoneuron loss is first apparent at ~60 days of age when 9 to 14% of the motoneurons are lost from the various tracts in the spinal cord (Zang and Cheema, 2002). By 110 days of age in the $SOD1^{G93A}$ mice, approximately half the motoneurons have been lost in the corticospinal, bulbospinal and rubrospinal tracts (Zang and Cheema, 2002).

### 1.3.4 Effects of gender on the $SOD1^{G93A}$ mouse model of ALS

As in human ALS patients, the disease progression is affected by gender in $SOD1^{G93A}$ mice. The onset of disease is delayed by ~21 days in female $SOD1^{G93A}$ mice as compared to male $SOD1^{G93A}$ mice, and the survival of the female mice is ~4 days longer (Veldink et al., 2003). The difference in survival time can be abolished by removing the ovaries of female $SOD1^{G93A}$ mice and hence reducing the exposure of female mice to estrogen (Groeneveld et al., 2004). The affects of the ovariectomy can be reversed by administering a high dose of the estrogen derivative 17-beta-estradiol, suggesting that the female sex hormone has protective effects in ALS (Groeneveld et al., 2004). Indeed *in vitro* estradiol protects motoneurons cultured from $SOD1^{G93A}$ mice against increased oxidative stress and glutaminergic exctitoxicity (Kruman et al., 1999).

Unfortunately, estrogen replacement therapy did not appear to confer protection the motoneurons of human ALS patients, and no benefit was reported in an epidemiological study of estrogen replacement therapy in women ALS patients (Rudnicki, 1999).

### 1.3.5 Exercise effects disease progression in the SOD1$^{G93A}$ mouse

Another factor that appears to have an affect on disease progression in the SOD1$^{G93A}$ mice is exercise. When the mice are allowed to exercise on running wheels their survival time is significantly prolonged as compared to normally caged SOD1$^{G93A}$ mice (Kaspar et al., 2005). Interestingly, the effects of exercise appear to be dependent upon both the intensity and duration of activity. The greatest increase in lifespan was reported after the SOD1$^{G93A}$ mice were allowed free access to running wheels from 40 days of age onwards (Kaspar et al., 2005). In contrast, when mice were forced to do high intensity exercise for 45 minutes a day on treadmills, the onset of disease was hastened in male mice (Mahoney et al., 2004). The negative effects of forced exercise were not seen when SOD1$^{G93A}$ mice of both genders were made to exercise at lower intensities (Veldink et al., 2003;Kirkinezos et al., 2003). For the most part these exercise studies focused on measures of survival and strength in the SOD1$^{G93A}$ mice, but did not address changes in the spinal cord or peripheral nervous systems. Kaspar et al. (2005) did report that voluntary exercise attenuated the loss of motoneurons from the spinal cords of SOD1$^{G93A}$ mice, but the mechanisms underlying this saving affect have not yet been explored. As voluntary exercise

causes a decrease in the excitability of some lower motoneurons (Beaumont and Gardiner, 2002) it is possible that exercise confers protection on motoneurons against excitotoxicity. It remains to be seen whether exercise does indeed prevent a die-back of motoneurons resulting in the saving of functional motor units.

### 1.3.6 Evidence of motor unit loss in the SOD1$^{G93A}$ mouse model

The clearly defined timeline of pathological events established by anatomical studies is contrasted by the ambiguous findings of functional motor unit studies (Fig 1-6). The first two studies that enumerated functional motor units in a hindlimb of a SOD1$^{G93A}$ mouse muscle recorded MUAP using an EMG that was inserted into the gastrocnemius muscle. One study found that the number of motor units was significantly less in SOD1$^{G93A}$ mouse medial compartment of the gastrocnemius muscles as compared to wildtype control mice at 47 days of age (Kennel et al., 1996). Although they did not graphically illustrate the time course of motor unit loss from the SOD1$^{G93A}$ mouse medial gastrocnemius muscle, Kennel et al (1996) showed that the CMAP declined linearly, in parallel with the alleged decline in motor unit number. The second study which used needle EMG to record potentials from the entire gastrocnemius muscle found that the compound muscle action potential did not decline until 60 days of age (Azzouz et al., 1997). Their findings indicated that the decline in CMAP and the parallel loss of motor units occurred in a biphasic manner, with an initial slow decline from 60 to 100 days of age, followed by a rapid decline in the

symptomatic phase of disease (Azzouz et al., 1997). More recently, Shefner and colleagues (1999) used surface EMG electrodes to record MUAPs from all the hindlimb muscles in the $SOD1^{G93A}$ mouse. Using surface EMG electrodes to enumerate functional motor unit numbers a linear loss from the extensor and flexor compartments that began at ~60 days of age was found (Shefner et al., 1999;Shefner et al., 2001).

The disagreement regarding the magnitude and time course of motor unit loss from these studies may be related to different methods used to enumerate motor units or to the differences in the muscle properties. It is unlikely, however, that differences in the motor unit number estimation methods would account for the discrepancies found in the number of motor units because Azzouz et al (1997) and Kennel (1996) reportedly used identical methods. Shefner et al., (1999) employed a slightly different method in that surface electrodes were used to record potentials from all lower hind limb muscles. These methods were verified in a later paper where two different types of MUNE protocols were used with very similar results (Shefner et al., 2002). It is more likely that the differences in the number of motor units estimated in these methods originate from preferential denervation in some muscles, but not others. Preferential denervation of fast twitch muscle has been reported using anatomical methods in which the motor endplates were mapped in conjunction with muscle fiber phenotypes in the triceps surae muscle group (Frey et al., 2000). Changes in a certain subpopulation of muscles may be masked when the population of motor units is very large, as when all the motor units are recruited in the gastrocnemius

(Azzouz et al., 1997) or in all the lower hind limb muscles (Shefner et al., 1999). Only one study thus far has measured the contractile force in an isolated hindlimb muscle; Derave (Derave et al., 2003) found that the force from the EDL muscle selectively declined after 90-days but the soleus did not, even in late disease. Measurements of force are needed to determine whether there is actually a loss of functional motor units, and to compare with previous EMG studies to determine whether there is dissociation.

## 1.4 THE OBJECTIVES AND AIMS OF THE PHD THESIS

The primary **aim** of the present PhD thesis was to establish the time course of motor unit loss and functional compensatory processes in the $SOD1^{G93A}$ mouse model of ALS, and to determine how these progressive functional changes may be modulated. To this end our **first objective** was to determine the extent of motor unit loss in the $SOD1^{G93A}$ mouse at a time point when there is already anatomical evidence of die-back, but the mice do not display overt signs of disease. We hypothesized that in asymptomatic mice compensatory processes including collateral axonal sprouting may mask the degeneration of lower motoneurons. Our **second objective** was to establish the time course of functional motor unit loss in four morphologically distinct hindlimb muscles of the $SOD1^{G93A}$ mouse. Enumerating motor units in four isolated muscles allows us to determine the functional consequences of preferential denervation of fast-twitch muscle fibers (Frey et al., 2000).

Our **third objective** focused on determining which factors may influence motor unit loss in the $SOD1^{G93A}$ mouse. As there are gender-dependent effects on disease onset in both the $SOD1^{G93A}$ mice and human ALS patients, we first explored whether gender influenced the rate or extent of motor unit loss in the mice. Activity also has dose-dependent effects on the lifespan of $SOD1^{G93A}$ mice so we explored the effects of voluntary wheel running exercise on motor unit properties. To differentiate between gender mediated differences in activity levels, activity was also imposed upon $SOD1^{G93A}$ mice by partially denervating the hindlimb muscles through spinal root avulsion.

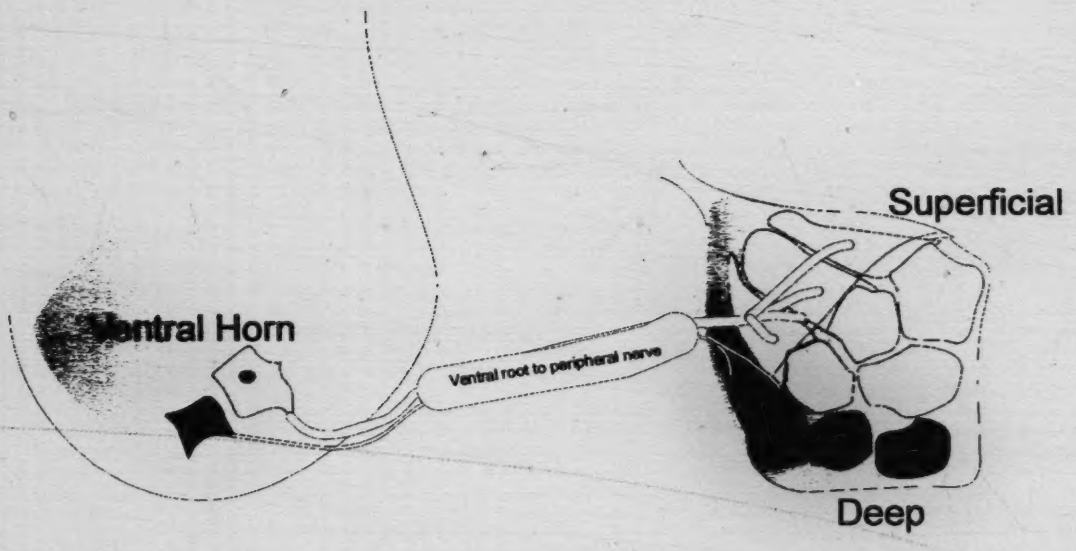


**Figure 1-1** The motor unit is made up of the axon of one α-motoneuron originating from the ventral horn of the spinal cord and all the muscle fibers that it innervates. The excitability of the motoneuron is determined by its size, synaptic inputs and electrophysiological properties. Smaller motoneurons, which project onto slow-twitch "red" muscle fibers in the deep regions of the muscles, are the most excitable because of their higher membrane resistance and lower rheobase. The larger motoneurons are less excitable, and are recruited when greater amounts of force are needed. These less excitable, large motoneurons innervate larger, more forceful, fast-twitch "white" muscle fibers in the superficial portion of the muscles.

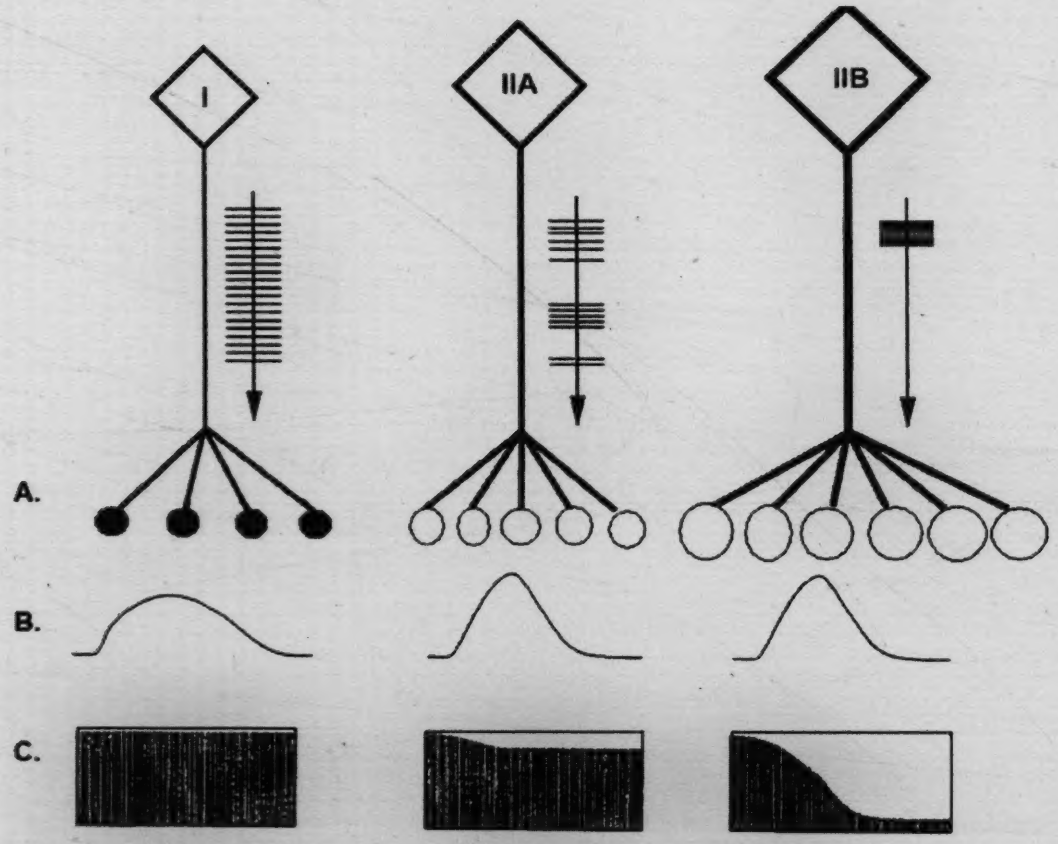


**Figure 1-2** Illustration demonstrating the matching between motoneuron and muscle fiber properties. *A)* The three main motor unit types are represented, with the motoneurons labeled according to the type of muscle fiber they innervate (type I= slow motor units, types IIA and IIB = fast m motor units. Horizontal lines represent the activity that is transmitted. Smaller, more excitable motoneurons innervate less muscle fibers than the larger motoneurons. *B)* A single muscle twitch typically produced by each motor unit demonstrates the differences in the time course of muscle contraction. *C)* Representative fatigue tests, with the horizontal lines representing activity.

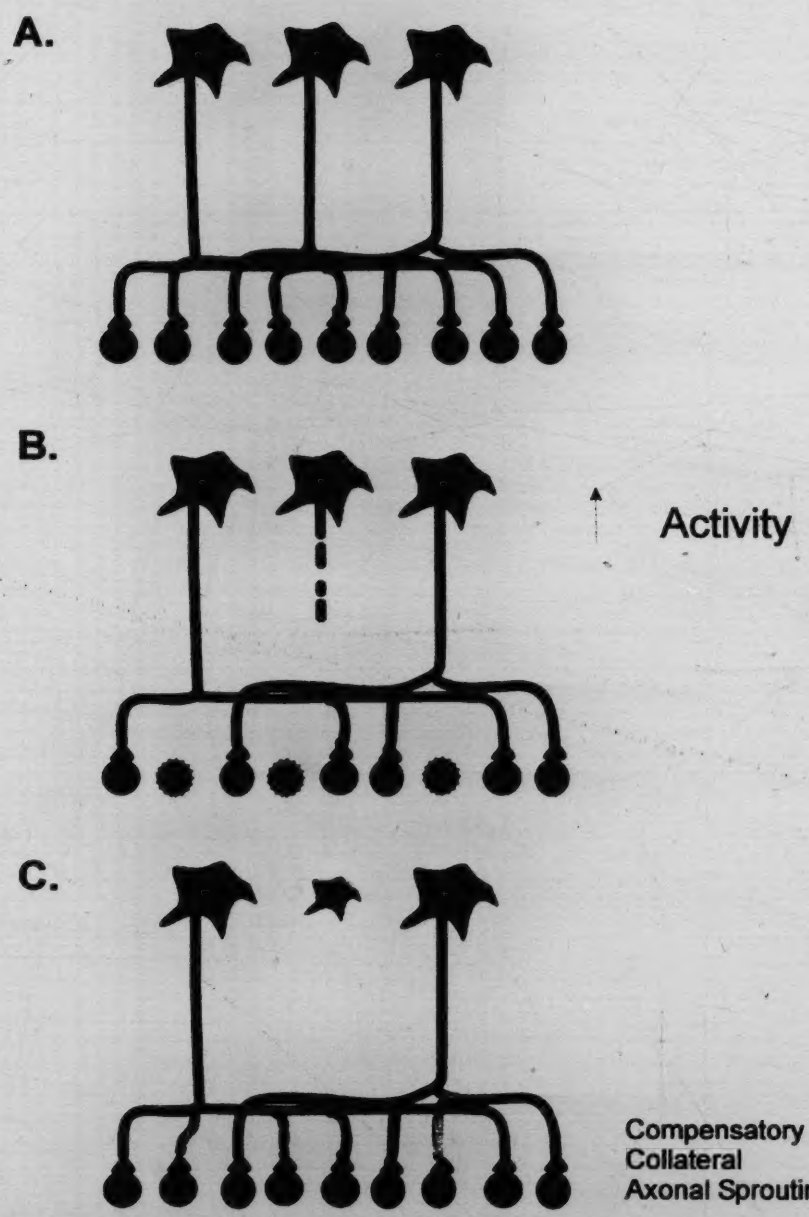


**Figure 1-3** Motoneuron die-back results in the denervation of muscle fibers, but it can be compensated for by an increase in the activity of surviving motoneurons *(B)* or through compensatory collateral sprouting *(C)*. *(A)* Motoneurons innervate fibers dispersed throughout the muscle, resulting in a characteristic "mosaic" pattern. *(B)* The loss of a motoneuron, through die-back or injury, leaves muscle fibers denervated, and compromises the force producing capacity of the muscle. In an effort to compensate for motor unit loss the remaining motor units increase their activity in order to generate more force. *(C)* Motor axons are also capable of sprouting new axon branches which are guided to denervated muscle fibers by specific molecular markers. The reinnervated muscle fibers than take on the characteristics of the motoneuron they are innervated by.

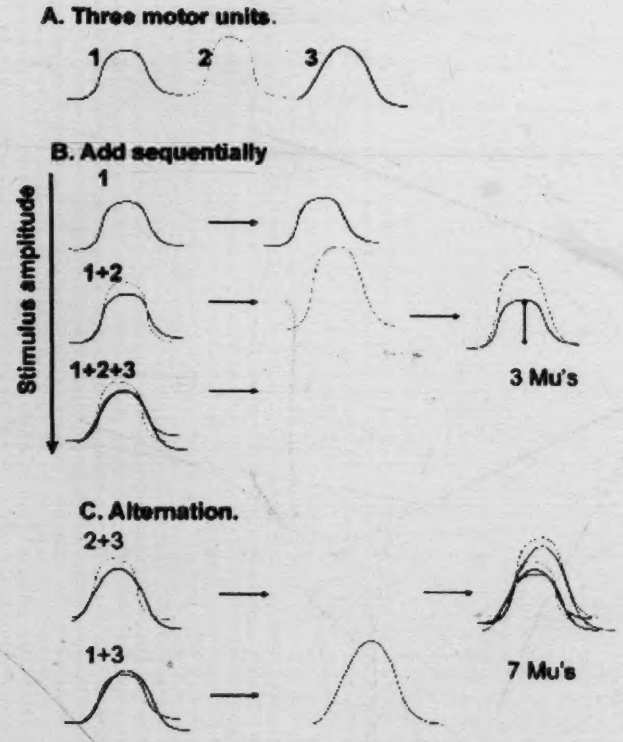


**Figure 1-4** Alternation can cause the overestimation of motor units. *(A)* The reliable recruitment of all the muscle fibers innervated by each motoneuron produces an all-or-none twitch. The forcefulness of this twitch can be quantified and is graphically illustrated here. *(B)* The incremental method of MUNE relies on the assumption that all motor units add in a reproducible, sequential manner so that the progressive recruitment results in incremental increases in the while muscle twitch force. *(C)* Alternation describes the phenomena whereby motor units with similar thresholds add in multiple ways, causing the number of increments to be overestimated.

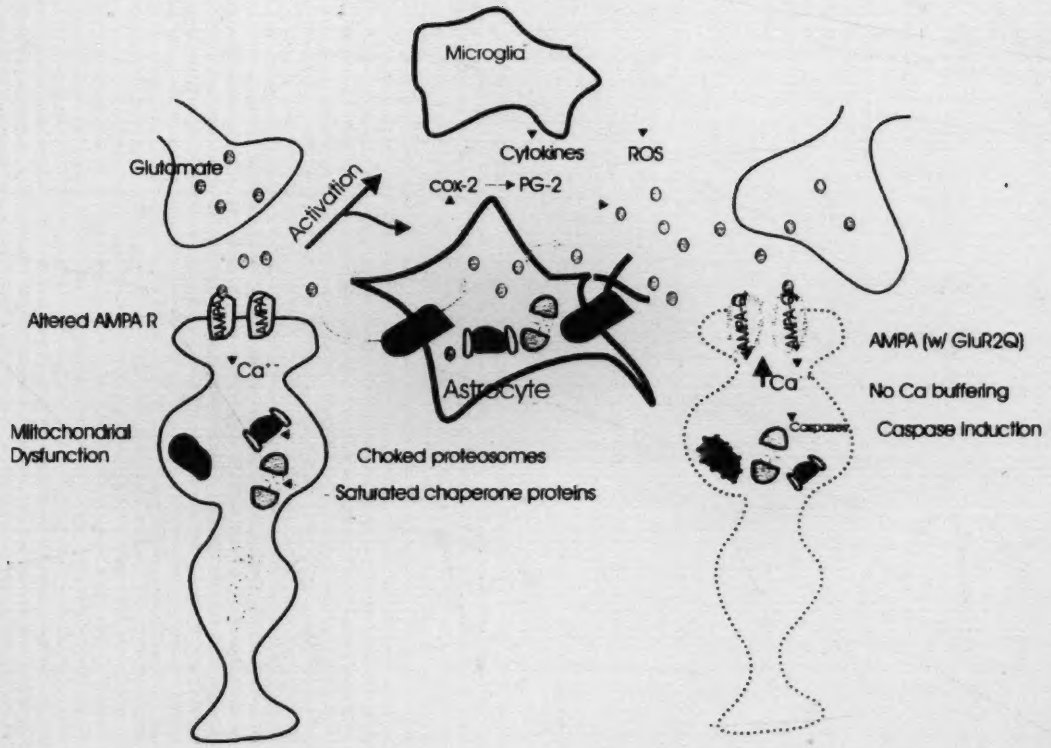


**Figure 1-5** Motoneuron degeneration in ALS is associated with the recruitment of numerous diverse pathways. What initiates the progressive degeneration has not yet been determined, but a current hypothesis postulates that ALS-associated mutations in SOD saturate chaperone proteins and proteosomes and hence "strangulate" the long axon processes of motoneurons. Adapted from Cleveland and Rothstein (2001).

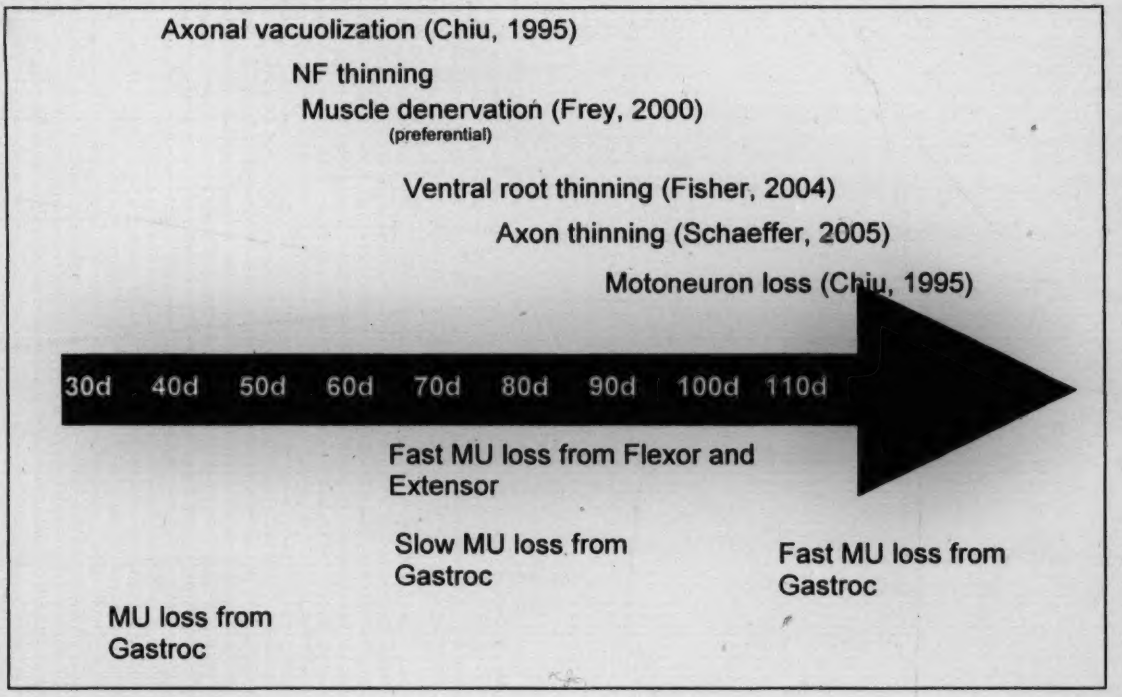


**Figure 1-6** Anatomical studies have established a clear time course of events associated with the motoneuron degeneration in the $SOD1^{G93A}$ transgenic mouse model of ALS. Measures of functional motor unit loss have not produced such unequivocal results.

**Table 1-1** A summary of the most popular motor unit number estimation methods used to study functional loss after injury and disease (Doherty et al., 1995;Daube, 1995;McComas, 1995)

| Method | Protocol | Assumptions | Advantage | Disadvantages |
|---|---|---|---|---|
| **Manual Incremental Stimulation**<br><br>*(McComas et al., 1971)* | - successive manually adjusted electrical stimuli are applied to nerve to elicit incremental "all-or-none" increments in muscle isometric force or CMAP | - each increment represents the addition of 1 motor unit<br>- ability to evoke single motor axons in an "all-or-none" manner<br>- thresholds of axons sufficiently separate so that they will be recruited in a reproducible and exact order<br>- excited motor units are representative | - reproducible<br>- non-invasive<br>- can be automated to prevent sampling bias | - alternation<br>- selection bias<br>- inaccurate when MUAP's or motor unit twitch forces are very small |
| **Multiple Point Stimulation**<br><br>*(Doherty and Brown, 1993)* | - the motor axon is stimulated along several sites<br>- at each site the first MUAP to be excited is recorded. | - at threshold, the nerve stimulation recruits only one motor axon reliably<br>- the sampled MUAP's are representive<br>- the sample size is large enough for meaningful and reliable methods | - the average SMUAP is based on actual, generated action potentials, and not a statistical estimate<br>- no alternation<br>- low stimuli amplitudes well tolerated by subjects<br>- can also study neuromusc transmission | - because the lowest threshold motor units are always selected this sampling method is not entirely random<br>- same motor unit may be excited multiple times |
| **F-Response Method**<br><br>*(reviewed by Doherty & Brown, 1995)* | - the F-waves are elicited after the CMAP is evoked by a train of stimuli<br>- F-wave is assumed to be one motor unit | - the F-wave is representative of all the SMUAP's in the muscle<br>- Independent of the stimulus intensity | - no alternation<br>- algorithm matching can be used to assure that each F-wave is unique<br>- latency can be determined<br>- lower intensity stimuli are better tolerated by subjects | - requires 200-300 CMAP's at ~30% max M-wave for ~10 F-wave responses<br>- chance of f-waves is higher in some pathological conditions, including ALS<br>- software required to assure uniqueness of f-waves |
| **Spike Triggered Averaging**<br><br>*(reviewed by Doherty & Brown, 1995)* | - subjects perform weak voluntary isometric contractions<br>- surface EMG recording is matched to recordings from a needle electrode, which is used to select single spikes -SMUAP's from >10 MU's | - spikes are generated one at a time | -can be used in isometric contractions, stretch & dynamic mvt.<br>- no alternation<br>- info collected about MU firing patterns and MU density<br>- distal or proximal muscles (don't need access to nerves)<br>- measure recruitment thresholds | - synchronized firing may distort the measurements of MU size<br>- needle electrode is uncomfortable<br>- cooperation is required to maintain contraction<br>- biased towards low threshold MU's |
| **Decomposotion Enhanced Spike Triggered Averaging**<br><br>*(reviewed by Doherty & Brown, 1995)* | - voluntary contraction for 20-60s<br>- algorithm is used to decompose the EMG into MUAP trains which are then used to trigger spike triggered averaging (see above) | - within a train of MUAP's that are excited during voluntary contraction the MUAP's maintain their distinct shape as long as the electrode is in the same position<br>-a small needle is needed to insure that 2 or more MUAP's will not be transposed | - increased number of MUAP's from one site<br>- MU's with higher thresholds can also be studied<br>- using an intramuscular needle allows the measurement of fiber densities and neuromuscular jitter | - the complexity of the EMG interference patterns makes it increasingly difficult to resolve shapes and peaks associated with individual SMUAP's<br>- the onset of SMUAP's cannot be determined and therefore MU number estimates must be derived from peak to peak measures |
| **Statistical**<br><br>*(Daube, JR, 1995)* | > 30 submaximal stimuli are given through nerve stimulation<br>- inherent variability of thresholds give intermittent firing | - in a Poisson distribution the variance of the 30 measures equals the size of the individual SMUAP's. | - can be used when the SMUAP's are very small<br>- doesn't require each motor unit to be identified | - time consuming<br>- a distribution that is normal or skewed to the right will give an incorrect estimate |

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## Chapter 2

# SELECTIVE LOSS OF THE MOST FORCEFUL MOTOR UNITS IN THE PRE-SYMPTOMATIC SOD1-G93A MOUSE MODEL OF ALS

## 2.1 INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease characterized by progressive and preferential loss of motoneurons (Cleveland and Rothstein, 2001). Approximately 10% of ALS cases are familial in origin, and of these, a further 20% have been linked to mutations in the cytosolic antioxidant enzyme, Cu/Zn superoxide dismutase (SOD1) gene (Rosen et al., 1993). When mutant human SOD1 is over-expressed in transgenic mice they develop a stereotypic syndrome. Expression of human mutant SOD1 with a glycine to alanine conversion at the 93$^{rd}$ codon (SOD1$^{G93A}$) results in motoneuron loss from the lumbar spinal cord, coincident with the onset of tremors and hindlimb weakness at 90 days of age (Gurney et al., 1994;Chiu et al., 1995). Anatomical studies have delineated a time-dependent dying-back of motoneurons with axon loss from ventral roots at 47 days of age (Fischer et al., 2004) and progressive denervation of hindlimb muscles (Frey et al., 2000). Attempts to enumerate the number of innervated motor units in pre-symptomatic mice with electromyography (EMG) have provided contradictory findings. A linear loss of functional motor units from the medial gastrocnemius (MG)

beginning at 47 days of age (Kennel et al., 1996) contrasts with the biphasic loss of gastrocnemius motor units significant only after 60 days of age reported in a later study (Azzouz et al., 1997). The only recording of muscle contractile force in pre-symptomatic mice demonstrated that the reduction in whole muscle force in extensor digitorum longus (EDL) did not precede the onset of symptoms at 90 days of age (Derave et al., 2003).

Without force recordings from the whole muscle and its single motor units we cannot discern whether the motor unit force rises in association with an increased number of muscle fibers per motor unit (innervation ratio; IR). The IR and the motor unit force are expected to increase in parallel due to the collateral sprouting of remaining motor axons which would compensate for partial denervation and maintain normal muscle force levels (Gordon et al., 1993). An anatomical study that mapped muscle fibers and their associated motor endplates and axons demonstrated that there was preferential denervation of fast-twitch fibers in the triceps surae muscle of the $SOD1^{G93A}$ mouse with coincident compensatory sprouting of the axons innervating slow-twitch muscle fibers at 50 days of age (Frey et al., 2000). However, Frey et al. (2000) did not calculate the IR of surviving motor units to determine whether this sprouting effectively increased motor unit size. The early loss of motor units from the MG muscle reported by Kennel et al. (1996) did appear to be partially compensated for by a small increase in the amplitude of the motor unit action potential, but similar increases were not seen by Azzouz et al (1997). To date the limited studies in which both EMG and force recordings were made from single motor

units showed that unitary EMG amplitudes are increased but not single motor unit forces in symptomatic human ALS patients (Milner-Brown et al., 1974;Vogt and Nix, 1997).

In this study we use isometric force recordings from 2 hindlimb muscles in the $SOD^{G93A}$ transgenic and the wild type $SOD^{WT}$ control mice to measure whole muscle and single motor unit contractile forces. In conjunction with our physiological examinations, we use immunohistochemistry to determine the number and type of the muscle fibers that are innervated. Thereby we investigated 1) whether the number of functional motor units in fast-twitch muscles of the hindlimb are reduced in pre-symptomatic $SOD^{G93A}$ transgenic mice as compared to the $SOD^{WT}$ control mice, 2) whether there is compensatory enlargement of the remaining motor units, and 3) which of the 3 factors that determine motor unit force are affected by the disease: IR, fiber cross-sectional area (CSA) and fiber specific force (SF).

## 2.2 METHODS

### 2.2.1 $SOD1^{G93A}$ and $SOD1^{WT}$ mice

Transgenic mice expressing a high copy number of the glycine to alanine base pair mutation at the 93$^{rd}$ codon of the cytosolic Cu/Zn superoxide dismutase (SOD1) gene ($SOD1^{G93A}$; B6JSL-TgN (SOD1-G93A)) were obtained from Jackson Laboratories, USA. The transgenic male $SOD1^{G93A}$ mice were bred to non-transgenic B6JSL hybrid females, and resulting progeny was identified using standard PCR protocol for the human SOD1 (Rosen et al., 1993) performed on ear biopsy samples taken at the time of weaning (approximately 21 days of age). The mice were identified using ear punches, and kept in standard animal housing with free access to water and standard rodent chow. Non-transgenic wild-type littermates were used as age matched controls ($SOD1^{WT}$). All of the experiments were carried out in accordance with Canadian Council for Animal Care and the University of Alberta Health Sciences Laboratory Animal Ethics committee approved all experimental procedures.

$SOD1^{G93A}$ mice become symptomatic at approximately 90 days of age. Symptoms included fine shaking, tremors and spasticity in the hind-legs (Chiu et al., 1995). Complete paralysis of the hindlimbs occurs within 30 to 40 days of initial symptom onset. The $SOD1^{WT}$ mice have not been reported to develop ALS-like disease (Gurney et al., 1994). The TA muscle was isolated and studied in a total of 4 $SOD1^{G93A}$ and 4 $SOD1^{WT}$ mice at 60-days of age, a time point that was approximately a month before the onset of symptoms. To confirm that motor units were lost from fast-twitch hindlimb muscles, we also enumerated motor

units in a fast-twitch antigravity muscle, the MG at 60-days of age. Again, we studied a total of 4 $SOD1^{G93A}$ and 4 $SOD1^{WT}$ mice, with further experiments being unnecessary as the results were statistically significant.

### 2.2.2 Electrophysiological studies

*Surgery* Mice were anaesthetized with an intra-peritoneal (IP) injection of a cocktail made up of Ketamine (100 mg/mL), Atravet (10 mg/mL) in sterile saline, at a dosage of 17.5mL/kg body weight. At regular intervals, additional anesthetic was administered IP to sustain surgical anaesthesia. The body temperature of the mice was maintained using a heat lamp, and saline was administered subcutaneously at 45-minute intervals. A laminectomy was done to isolate the L4 and L5 ventral roots. The TA or the MG muscle tendons were isolated bilaterally and tied with a 4.0 silk thread for attachment with 4.0 silk to a strain gauge (Kulite model KH-102). Two silver wire electrodes were sutured alongside the sciatic nerves for stimulation (Fig 2-1*A*). Both hindlimbs were prepared in the event of any problems in root dissection or hindlimb immobilization. One hindlimb was immobilized by clamping the knees and the ankles, while being careful not to interfere with the blood supply to the muscles. A paraffin pool was fashioned to dissect and tease the ventral roots and elicit motor unit forces.

*Isometric recordings of muscle and motor unit contractile force* Evoked isometric forces were amplified and visualized on an oscilloscope and digitized using Axoscope Software (Version 8.0, Axon Instruments, USA). Muscle length was

adjusted for maximal isometric twitch force in response to stimulation of the sciatic nerve. Whole muscle twitch and tetanic forces were recorded in response to single and repetitive suprathreshold (twice threshold amplitude) stimulation of the sciatic nerve at 0.5 and 100Hz, respectively (Fig 2-1*A*).

To determine the average force produced by motor units, we teased ventral roots into fine filaments. Beginning at a stimulus voltage of 100μs duration that was below the threshold for recruitment of motor units, we applied a manually controlled graded electrical stimulus of 0 to 10V to the ventral root filaments at a frequency of 0.5 Hz. The resulting all-or-none incremental increases in isometric force were recorded. We then gradually raised the voltage manually and progressively repeated the method outlined above to stimulate all the motor axons within the isolated filament. At least 5 filaments were teased for stimulation in order to recruit and record twitch force contractions from at least 50% and as many as 100% of the muscle units in the TA and MG muscles of the $SOD1^{G93A}$ and $SOD1^{WT}$ mice. Motor unit number and mean twitch force were obtained by overlaying the digitized twitch forces in Clampfit Software (Version 8.0, Axon Instruments, USA) and template subtraction of the forces to give the individual motor unit twitch forces. The average motor unit force was divided into the maximal isometric twitch force to give an estimate of the number of motor units, as previously described in human (McComas et al., 1971) and rodent muscles (Fu and Gordon, 1995; Fig 2-1*B*).

### 2.2.3 Muscle fiber type determination for the TA muscle

*Muscle collection and sectioning* The TA muscles were carefully removed immediately after the force measurements were completed. Muscles were then embedded in cutting medium (Tissue-Tek Optimal Cutting Medium Compound, Miles Scientific, USA) and frozen in melting isopentane cooled in liquid nitrogen (-156 °C). Samples were stored at –80 °C until sectioning. Serial 12μm-thick sections were cut at –25°C and collected onto glass Superfrost Plus slides (Fisher Scientific, California, USA), air dried for one hour, and stored at –80°C until immunohistochemical staining.

*Immunohistochemistry for Myosin Heavy Chain Isoform, Neural Cell Adhesion Molecule and Dystrophin* Immunostaining was completed according to previously published protocols (Putman et al., 2003;Martins et al., 2006). In brief, prior to treatment with antibodies the sections were washed in phosphate-buffered saline (PBS) with 0.1% Tween-20 (PBS-T), with PBS, and then incubated for 15 minutes in 3% $H_2O_2$ in methanol. Sections were stained for either myosin heavy chain (MHC) I isoform (clone BA-D5), MHCIIa (clone SC-71), all MHC's except MHC IId/x (clone BF-35), neural cell adhesion molecule (NCAM) or dystrophin. Sections were then incubated at room temperature for 1 hour in a blocking solution (BS-1: 1% bovine serum albumin, 10% horse serum in PBS-T, pH 7.4) containing Avidin-D Blocking Reagent (Vector Laboratories Inc.). Sections that were to be stained for MHCIIb (clone BF-F3) or NCAM were incubated at room temperature for 1 hour in a similar blocking solution, with the exception that goat serum was substituted for horse serum (BS-2). The primary antibodies were

diluted in the appropriate blocking solutions that also contained Biotin Blocking Reagent (Vector Laboratories Inc.), overlaid onto sections and incubated overnight at 4°C. The dilutions of the primary antibodies were: BA-D5, 1:400 in BS-1; SC-71, 1:100 in BS-1; BF-35, 1:10,000 in BS-1; BF-F3, 1:400 in BS-2; DYS2, 1:10 in BS-1; NCAM 1:400 in BS-2. Sections were washed as before and either biotinylated horse-anti-mouse-IgG (1:200, Vector Laboratories; for BA-D5, SC-71, BF-35 and DYS2), biotinylated goat-anti-rabbit Ig-G (1:400; for NCAM) or biotinylated goat-anti-mouse-IgM (1:200, Vector Laboratories; for BF-F3) was applied for 1 hour at room temperature. Sections were then washed and incubated with Vectastain ABC Reagent (avidin-biotin Horse Radish Peroxidase (HRP) complex) for one hour, followed by repeated washes. Finally, immunoreactivity was detected by exposing sections to a solution containing 0.07% diaminobenzidine, 0.05% $H_2O_2$, and 0.03% $NiCl_2$ in 50 mM Tris-HCl (pH 7.5). The reaction was stopped by rinsing with distilled $H_2O$. Sections were then dehydrated in ethanol, cleared in xylene and mounted using entellan (Darmstadt, Germany).

*Immunohistochemical Analyses* Muscle fiber cross sectional areas (CSA) and fiber types were quantified over the entire cross section of 4 $SOD1^{G93A}$ and 4 $SOD1^{WT}$ TA muscles; a total number of 13,924 fibers were analyzed. Pictures of the entire TA cross sections were taken with Leitz Diaplan microscope (Enrst Leitz Wetzlar GmbH, Germany) fitted with a Pro Series High Performance CCD camera (Media Cybernetics, USA), and analyzed using a custom designed

analytical imaging program (Putman et al., 2000). Fiber types were identified by the staining of MHC isoforms; type I fibers stained for MHCI, type IIA for MHCIIa and type IIB fibers for MHCIIb. Type IID/X fibers were identified as those that remained unstained by clone BF-35 but stained for all other anti-MHC antibodies. These same fibers were also negative for immunoreactivity with all other anti-MHC antibodies (Fig 2- 2).

### 2.2.4 Statistical analysis

The data are presented as arithmetic means $\pm$ standard errors (SE). Statistical significance between groups was assessed through Students' t-test in Sigma Plot (SPSS, Version 8.0). Differences were considered statistical significant if $p<0.05$. Distributions were tested for normality using a one-sided Kolmogrov-Smirnov test, and for non-normally distributed data, significant differences were determined using the Mann-Whitney U test (SPSS Version 14.0, 2005). Power calculations were made to confirm sufficient power and/or sample size using PC-Size (STATOOLS, Version 2.13, 1986). Power values of at least 0.8 were required to consider results significant.

## 2.3 RESULTS

### 2.3.1 Motor unit force declines in the TA muscle of SOD1$^{G93A}$ mice before the onset of overt weakness

SOD1$^{G93A}$ male mice were studied at 60 days of age when they do not yet exhibit clinical signs of weakness, but there is anatomical anatomical evidence of axon die-back, which includes axon loss (Fischer et al., 2004) and denervation of fast-twitch muscle fibers (Frey et al., 2000). Collateral axonal sprouting can compensate for partial denervation in normal mammalian muscle by increasing the number of muscle fibers per motoneuron (the IR of motor units) and thereby the corresponding motor unit force (Gordon et al., 1993). To determine whether muscle contractile force is maintained by the enlargement of remaining intact motor units in the SOD1$^{G93A}$ mouse model of ALS, ventral root filaments were isolated to evoke isometric twitch force of single motor units in the TA muscle of 60-day old SOD1$^{G93A}$ (n=4) and SOD1$^{WT}$ (n=4) mice. Normal control values were established by recording the force from 65 $\pm$ 6 motor units (mean $\pm$ SEM) in each of the SOD1$^{WT}$ mouse TA muscles. The frequency distribution of the motor unit forces is shown in Figure 2-3*A*. The range of motor unit forces in the SOD1$^{WT}$ mouse TA muscles was 13 $\pm$ 2mN, with the forces being skewed towards the higher values (Fig 2-3*A*). Approximately 70% of the motor units produced force within 0.5 to 4.0mN, while the remaining motor units (~30%) produced higher forces (4.0-18.9mN). This skewed distribution, with a higher proportion of small force-producing motor units, is typical of motor unit distributions in mammalian

skeletal muscles including rats (Tötösy de Zepetnek et al., 1992) and cats (Rafuse et al., 1997).

Twitch forces of 33 ± 10 motor units were recorded in each of the $SOD1^{G93A}$ mouse TA muscles at 60 days of age. The range of motor unit forces was 3.6 ± 1.4 mN in the $SOD1^{G93A}$ mouse TA, which was significantly less than the range in the $SOD1^{WT}$ mice of 13 ± 2mN ($p < 0.001$). The right skewed distribution was also less pronounced in the $SOD1^{G93A}$ mouse TA because 90% of the motor units produced forces between 0.5 and 4mN, as compared to 70% in the control $SOD1^{WT}$ mouse TA muscles. The upper maximum of motor unit forces was only 8.1mN in the $SOD1^{G93A}$ mouse TA muscle, compared with 18.9mN in the $SOD1^{WT}$ mouse muscle (Fig 2-3). The decreased proportion of the most forceful motor units in the $SOD1^{G93A}$ mouse TA muscles and the consequent significant reduction in range is well illustrated by comparing the cumulative frequency histograms of the motor unit twitch forces in the $SOD1^{G93A}$ and in the $SOD1^{WT}$ TA muscles (Fig 2-3*C*). The leftward shift of the histograms in the $SOD1^{G93A}$ mice is statistically significant ($p<0.001$) with a disproportionate decline in the proportion of the most forceful motor units. The reduced proportion was indicative of either preferential loss of the largest, most fatigable motor units or a reduction in the contractile force developed by the surviving motor units in the $SOD1^{G93A}$ mouse. Previously reported pre-symptomatic motor axon die-back, as evidenced by anatomical studies (Fischer et al., 2004), was therefore not compensated for by an increase in the average force developed by the surviving motor units in the $SOD1^{G93A}$ mouse.

### 2.3.2 The parallel decline in average motor unit force and number caused a reduction in whole muscle force in the pre-clinicial phase of disease in SOD1$^{G93A}$ mice

Functional muscle deficits have not been previously reported in pre-symptomatic SOD1$^{G93A}$ mice, with a deficit in the force developed by the fast-twitch EDL muscle reportedly occurring only after 90-days of age (Derave et al., 2003). Our findings of a reduced proportion of the most forceful motor units and anatomical findings of motor axon die-back (Fischer et al., 2004) predict that muscle force and the numbers of intact motor units will be reduced in pre-symptomatic SOD1$^{G93A}$ mice. However, muscle contractile force is a product of average motor unit force and the number of motor units (equation 1)

**(1)** Muscle force = motor unit force x number of motor units

Isometric twitch and tetanic contractile forces of the TA muscles were recorded in response to supra-maximal stimulation of the sciatic nerve in both SOD1$^{G93A}$ (n=4) and control SOD1$^{WT}$ (n=4) mice. Both twitch and tetanic forces were significantly lower in the SOD1$^{G93A}$ than in the SOD1$^{WT}$ mice ($p<0.01$; Fig 2-4*A,B*). The reduction in twitch force is further illustrated by representative tracings in the inset of Fig 2-4*A*. The whole twitch and tetanic muscle forces in the SOD1$^{G93A}$ were reduced in parallel by ~80% ($p<0.001$; Fig 2-4*AB*). This is the first report of a pre-symptomatic decline in the force produced by any muscle in the pre-symptomatic SOD1$^{G93A}$ mouse.

The number of motor units in the TA muscles of the SOD1$^{WT}$ and SOD1$^{G93A}$ mice was calculated according the method described by McComas

(1971) for human subjects and by Fu *et al.* (1992) for rodents. Comparison of the number of motor units in the muscles of the $SOD1^{G93A}$ and $SOD1^{WT}$ mice showed that there were 60% fewer units in the $SOD1^{G93A}$ mouse muscles, representing a significant pre-symptomatic loss of functional motor units (Fig 2-4*C*).

### 2.3.3 Motor unit force declines due to a decrease in the force produced per muscle fiber

The surviving motor units in the $SOD1^{G93A}$ mouse TA muscle develop less force on average than the motor units in the $SOD1^{WT}$ mouse TA muscle due to a reduction in the proportion of the most forceful motor units (Fig 2-3*C*). The reduced proportion of the latter motor units is due to either a selective loss of the largest motor units or reduced force developed by the surviving motor units. Motor unit force is determined by 2 factors: the number of muscle fibers per motor unit (IR) and the force produced per each fiber (equation 2)

**(2)** Motor unit force = IR x Force/Fiber

Previous mapping of muscle endplates and axons revealed that the motor axons that innervated slow-twitch muscle fibers preferentially extended collateral sprouts to reinnervate denervated muscle fibers (Frey et al., 2000). In contrast, longitudinal *in vivo* visualization of motor endplates and axons showed that IR only increases in late-stage, symptomatic disease in the $SOD1^{G93A}$ mice hindlimb muscles (Schaefer et al., 2005). We now have to estimate IR in order to determine whether motor unit force in the $SOD1^{G93A}$ mouse decreases due to a

change in the IR and/or changes in the amount of force produced per muscle fiber.

To determine average IR all innervated muscle fibers were counted in the $SOD1^{G93A}$ mouse TA muscle and divided by the number of motor units (equation 3).

**(3)** IR = number of muscle fibers/ number of motor units

NCAM immunohistochemical staining was used to identify NCAM-positive denervated muscle fibers. In the $SOD1^{WT}$ mouse TA muscle there were no muscle fibers that stained positive for NCAM (Fig 2-5*A)* while ~50% of the fibers in the $SOD1^{G93A}$ mouse TA muscle stained positive for NCAM (Fig 2-*5B)*. The muscle fibers in the superficial areas of the TA tended to be angulated, indicative of long-term denervation (Cullen et al., 1992). In the deeper regions of the muscle we also noted that the NCAM positive muscle fibers tended to sustain a hexagonal profile, indicating that they had only recently become denervated (Fig 2-5*B)*. These findings are consistent with a dynamic process of denervation in the muscles of the $SOD1^{G93A}$ mice where the earliest muscle fibers lose their innervation are in the superficial layer of the TA muscle.

Quantification of NCAM negative muscle fibers revealed that there were 2181 $\pm$ 140 innervated muscle fibers in the $SOD1^{WT}$ mouse TA muscle. In contrast, ~57% of the muscle fibers were NCAM positive in the $SOD1^{G93A}$ mouse TA muscle, and there were only 1242 $\pm$ 124 innervated muscle fibers (Fig 2-5*C)*. The average IR was calculated by dividing the numbers of muscle fibers by the number of intact motor units in each mouse. The IR was not significantly greater

in the SOD1$^{G93A}$ mouse muscles as compared to the SOD1$^{WT}$ mouse TA muscles ($p=0.23$). Since the IR was not significantly changed in the SOD1$^{G93A}$ mice, it follows that the force per fiber declines to account for the reduction in average motor unit force, according to equation 2. Force per fiber was calculated by the ratio of muscle force and the number of muscle fibers (equation 4).

**(4)** Force per fiber = whole muscle force/ number of innervated fibers

The innervated muscle fibers in the SOD1$^{G93A}$ mouse produced only 40% of the force produced by the muscle fibers in the SOD1$^{WT}$ TA muscles ($p<0.05$; Fig 2-5*D*). The decline in motor unit force was accounted for by reduction in force per fiber (equation 2).

**(5)** Force per fiber = Fiber CSA x Specific force

Because force per fiber is the product of fiber CSA and specific force (equation 5), we expect that either one or both of these variables were reduced in the SOD1$^{G93A}$ mouse.

### 2.3.4 Changing muscle fiber type proportions causes a shift in CSA distribution

To determine whether a reduction in muscle fiber CSA accounts at least in part for the reduced force-producing capacity of muscle fibers in the 60 day old SOD1$^{G93A}$ mouse TA muscles we measured the CSA of all innervated muscle fibers. The distribution of all innervated muscle fibers in the SOD1$^{WT}$ mouse TA was skewed towards the right with an average CSA of 1551.1 $\pm$ 9.1 $\mu m^2$ (Fig 2-6*A*). The right skewed distribution of the total CSA was due to the high

proportion (65%) of type IIB muscle fibers. The type IIB muscle fibers were the largest fibers in the mouse TA muscle, with a mean CSA of 1919.4 ± 10.8 $\mu m^2$ (Fig 2-6*B*). The remaining fibers in the TA muscle were smaller type IIA or IID/X fibers that comprised approximately 4% and 31% of the muscle fiber type proportions, respectively. The type IID/X and IIA fibers had mean CSA's of 939.0 ± 7.6 $\mu m^2$ and 482.4 ± 15.0 $\mu m^2$.

The total CSA of muscle fibers in the $SOD1^{G93A}$ mouse TA muscle at 60-days was also skewed towards the higher CSA values, but the proportions and average sizes of the muscle fibers were significantly different. The average CSA of all the muscle fibers was reduced by ~30% to 1088.5 ± 7.2 $\mu m^2$ ($p<0.001$; Fig 2-6*C*). The greatest proportion of muscle fibers were still type IIB (42%), but the average CSA of these fibers was reduced by ~39% to 1162.1 ± 12.9 $\mu m^2$ ($p<0.001$; Fig 2-6*D*). As these fibers were smaller, the distributions of the three different muscle fiber types overlapped to a greater extent in the $SOD1^{G93A}$ than in the $SOD1^{WT}$ mouse TA muscle. The proportion and size of the type IID/X and IIA fibers was significantly greater in the $SOD1^{G93A}$ than in the $SOD1^{WT}$ mouse TA muscle. Type IID/X muscle fibers accounted for 38% of the total fibers, and had an average CSA of 1112.4 ± 9.6 $\mu m^2$. The proportion of type IIA muscle fibers was increased to 14%, and these muscle fibers had an average CSA of 782.5 ± 13.1$\mu m^2$ (Fig 2-6*C*). The increase in the proportion of the type IIA fibers from 4 to 14% is indicative of conversion to slower fiber phenotypes within the subpopulations of fast-twitch fibers. This conversion is further evidenced by the

increase in mean CSA of the type IIA muscle fibers, which are the smallest type II fibers in normal, healthy mice (Fig 2-6*B*).

A small proportion of the muscle fibers also co-expressed two different MHC isoforms, which provided further evidence of ongoing muscle fiber type conversion. Using the present method wherein type IID/X was detected as the fibers that were left unstained when sections were stained with the BF-35 antibody, we were unable to detect type IIA/X or type IIB/X hybrid fibers. Only type IIA/IIB fibers could be detected (Putman et al., 2000).

The decrease in both the proportion and average size of the type IIB muscle fibers in the $SOD1^{G93A}$ mouse TA muscle, as compared to $SOD1^{WT}$ mouse TA muscles, accounted for the decrease in the average overall CSA. The average muscle fiber CSA reduced by ~29% in the $SOD1^{G93A}$ mice as compared to the $SOD1^{WT}$ mice TA muscles (Fig 2-6*D*), in contrast, the average force produced per muscle fiber was reduced by ~60%. As the reduction in fiber CSA does not account entirely for the reduction in force per fiber, there must also be a reduction in specific force of the muscle fibers (equation 5). The specific force of the fibers is the amount of force produced per unit area. In cats and rats, the specific force changes with muscle fiber type proportions and changes in the distribution of muscle type proportions may account for a reduction in the reduction in average force. In the mouse the specific force is proportional to the CSA, but not to the muscle fiber type fibers (Bottinelli et al., 1994;Bottinelli, 2001;Andruchov et al., 2004). Therefore, the changes in phenotypic proportions will not alter the specific force of muscle fibers. A decrement in force per fiber

independent of changes in the average CSA may be due to other disease-related factors, including alteration of the myofibril packing density, affecting the TA muscle fibers of 60 day old $SOD1^{G93A}$ mice. Changes in the specific force of $SOD1^{G93A}$ mouse muscle fibersare discussed further in the Discussion.

Changes in the phenotypic proportions will influence the force per fiber because they will cause a change in the average CSA. However, the CSA changes not only due to a decrease in the proportion of the largest type IIB muscle fibers, but also because the type IID/X and IIA muscle fibers hypertrophy while the type IIB fibers atrophy.

### 2.3.5 Preferential denervation causes a decrease in muscle fiber CSA

The CSA of the different muscle fiber types in the $SOD1^{G93A}$ mice and $SOD1^{WT}$ mice are summarized and compared in Fig 2-7*A*. As the fiber types are not distributed normally, we present the medians of CSA areas, and significance is tested using the Mann Whitney-U test. The type IIB fibers are significantly smaller in the $SOD1^{G93A}$ mice, while the type IIA and IID/X fibers displayed significantly larger CSA's (Fig 2-7*A*; $p<0.001$). Not only are the type IIB fibers smaller, but there were significantly fewer IIB fibers in the $SOD1^{G93A}$ as compared to the $SOD1^{WT}$ mice (Fig 2-7*J*). The decrease in the number and CSA of type IIB muscle fibers in the $SOD1^{G93A}$ mouse, as compared to the $SOD1^{WT}$ mouse TA muscles, accounted for the change in the muscle fiber type distributions of CSA as compared to the $SOD1^{WT}$ mouse muscles. The

preferential decrease in the proportion of innervated type IIB and IID/X muscle fibers was due to the selective denervation of these muscles. Conversion of muscle fiber phenotypes was evidenced by the co-expression of two different MHC isoforms in a small number of muscle fibers, and by the increase in the proportion and size of the type IIA muscle fibers (Fig 2-6). We speculate that the limited compensatory sprouting process, that prevents the IR from significantly changing, is also responsible for the small changes in the numbers of the IIA fibers. However, activity dependent conversion, due to an increase in the loading of remaining motor units, may also contribute to the conversion of type IIA muscle fibers to IID/X in the $SOD1^{G93A}$ mice.

### 2.3.6 Preferential functional motor units in an antagonistic muscle

To establish that muscle function does not influence the progressive loss of the largest motor units that was observed in the TA muscle, we recorded muscle and motor unit contractile forces from the antigravity MG muscle. The MG is part of the gastrocnemius muscle group, a group of fast-twitch muscles that are composed primarily of type IIB muscle fibers in the mouse (Hämäläinen and Pette, 1993) and which attach at the ankle to extend the foot. In control 60-day old $SOD1^{WT}$ mice the average motor unit force in the MG muscle was 3.9 $\pm$0.2 mN, with minimum and maximum forces of 0.8mN and 19.1mN, respectively (Fig 2-8*A*). As in the TA muscle (Fig 2-2) the motor unit forces in the $SOD1^{WT}$ mice were skewed, with a high proportion of less forceful motor units. In the pre-symptomatic $SOD1^{G93A}$ mouse MG muscle, the average motor unit force declined

to 1.9 ± 0.1 mN and the range of motor unit forces was 6.4 ± 2.8 mN, which was significantly less than in the $SOD1^{WT}$ mice ($p < 0.05$; Fig 2-8*B*). The lower range, observed both in the force produced by MG and TA muscles (Fig 2-2 and Fig 2-8, respectively) indicates that the most forceful motor units are lost in both muscles. In the MG muscle, the preferential loss of the largest motor units results in a ~80% decline in whole muscle twitch force (Fig 2-8*C*) and a parallel decline in tetanic force. Approximately 40% of the functional motor units remain intact in the MG muscle, as compared to the age matched control $SOD1^{WT}$ mice (Fig 2-8*D*). The similarities between the decline in muscle and motor unit forces in the TA and MG muscles in the $SOD1^{G93A}$ mice indicate that the preferential loss of the most forceful, fast-fatigable motor units and their composite type IIB muscle fibers accounts for the decline in muscle and motor unit forces.

## 2.4 DISCUSSION

This is the first report of a preferential loss of the most forceful motor units in the $SOD1^{G93A}$ mouse model of ALS. In pre-symptomatic mice we found that the early and selective loss of functional motor units is consistent with anatomical data of preferential die-back of the largest motor axons (Fischer et al., 2004), and the consequent denervation of the most forceful, type IIB muscle fibers (Frey et al., 2000). Our combined anatomical and electrophysiological approach allowed us to correlate reductions in average motor unit force (Fig 2-3) with an increased proportion of less forceful, smaller muscle fibers (Fig 2-6). The consequent reduction in average muscle fiber force caused a decline in motor unit force, despite an unchanged IR (Fig 2-5). The lack of a parallel change in the IR confounded the interpretation of published EMG studies, which relied on measurements of motor unit action potential size to enumerate functional motor units (Kennel et al., 1996;Azzouz et al., 1997;Shefner et al., 1999). Previously, a lack of correspondence in motor unit size, as measured by EMG, and the forcefulness of motor units was reported only in symptomatic ALS patients (Milner-Brown et al., 1974;Vogt and Nix, 1997).

The number of intact motor units in the fast-twitch TA and MG muscles is reduced by 60% in the 60 day old $SOD1^{G93A}$ mice and the surviving motor units were less forceful (Fig 2-3 to Fig 2-4; 2-8). In the TA muscle, the IR did not change significantly (Fig 2-5), and the reduction in average motor unit force was due to an increase in the proportion of smaller, type IIA and IID/X muscle fibers (Fig 2-6). The change in proportions of muscle fiber phenotypes was due to a

preferential loss of the most forceful motor units that included type IIB fibers (Fig 2-7) and to the sprouting of the axons innervating the type IIA muscle fibers, resulting in fiber type conversion. There was a trend for a compensatory increase in the IR of the remaining motor units. We speculate that the increase in the IR did not reach significance because characterization of muscle fiber types revealed an absence of type I and a low number of type IIA fibers. These two muscle fiber types are the only ones innervated by motoneurons that have previously been shown to exhibit a robust capacity for collateral axonal sprouting in the $SOD^{G93A}$ mouse (Frey et al., 2000). A trend for the IR to increase (Fig 2-5) indicates that there might be some sprouting from the motor axons that innervate type IIA muscle fibers. However, this increase could not be resolved with our measurements of IR because the proportion of type IIA fibers is very low in normal mouse TA muscles (Fig 2-.7; Hämäläinen and Pette, 1993). In addition to collateral axonal sprouting, muscle fiber type conversion may also occur due to changes in the recruitment pattern of motoneurons. Indeed, at 60-days of age, the surviving motor units in the TA muscle exhibit the adaptive potential for activity-dependent conversion of the relatively large type IIB fibers to the smaller type IIA and IID/X fibers (Fig 2-6).

Increasing the activity of motor units in normal rodents results in fiber type conversion that is first evidenced by a reduction in the type IIB fiber CSAs in rat muscles after 12 days of low frequency electrical stimulation (Delp and Pette, 1994). This daily stimulation is effective in converting muscle fibers progressively through IID/X, IIA to I types in conjunction with slowing of the muscle contractions

and conversion of motor units to the slow oxidative fiber type that contains type I MHC and oxidative metabolic enzymes in normal rats (Putman et al., 2004) and cats (Gordon et al., 1997). Here, we report a preferential decline in the CSA of type IIB muscle fibers in the 60-day old SOD1$^{G93A}$ mouse TA muscle (Fig 2-6,7) that parallels the preferential decline seen in conditions of experimentally increased motor unit activity.

Evidence of activity dependent fiber type conversion observed in the TA muscles of the 60-day old SOD1$^{G93A}$ mouse may be directly linked to the increased motoneuron activity that is associated with reported membrane hyperexcitability (Kuo et al., 2004) and the increased functional load on the reduced population of motor units. Aside from causing a size-dependent change in the forcefulness of motor units, activity- or load-dependent conversion should also change the sprouting competence of motor axons. Increased motor unit size seen in primarily fast-twitch muscles in longitudinal visualization of motor axons (Schaefer et al., 2005) or with EMG recordings in late stage of disease (Kennel et al., 1996;Shefner et al., 1999) occurs because the proportion of type IIA fibers that are innervated by axons with increased sprouting competence (Frey et al., 2000) rises due to both preferential die-back and activity-dependent conversion.

In normal mice, the specific force does not vary with muscle fiber types (Andruchov et al., 2004) and the differences in fiber CSA dictate the force-producing capacity of the muscle fibers (Stelzer and Widrick, 2003). If the specific force remains unchanged in the 60-day old SOD1$^{G93A}$ mouse, the

change in force produced per fiber should be proportional to the changes in CSA. However, a reduction in the average muscle fiber CSA of 29% (Fig 2-6) was not proportional to the 61% decrease in average muscle fiber force (Fig 2-5). Our findings therefore indicate that the specific force of muscle fibers was reduced in the 60-day old SOD1$^{G93A}$ mouse TA muscle. Although earlier data showed no loss of specific force in fast-twitch muscle fibers in the mouse at 60-days, the authors did not distinguish between the different type II fiber types (Atkin et al., 2005). As type II fibers were not uniformly susceptible, their comparative analysis of fibers from fast-and slow twitch muscles may have masked a preferential decline in the specific force of IIB fibers. Specific force may be reduced due to yet to be determined pathological changes in the muscle fibers.

Like Atkin et al (2005) we chose to study SOD1$^{G93A}$ at 60 days of age because they are reportedly asymptomatic (Chiu et al., 1995). This lack of symptoms is surprising considering the loss of ~80% of TA and MG muscle forces. Indeed, muscle specific tests do detect the early manifestation of preferential motor unit loss (Wooley et al., 2005). At 52 days of age the SOD1$^{G93A}$ mice have a reduced maximal running speed (Veldink et al., 2003). In normal rodents, motor units in the TA are progressively recruited with increased treadmill speeds (Roy et al., 1991), therefore a decrease in the ability to sustain running speed is consistent with a loss of TA motor units (Fig 2- 4).

From observations in the SOD1$^{G93A}$ mouse model of ALS we now postulate a hypothesis about the progressive changes in motor unit contractile properties. Our hypothesis readily accounts for the observed dissociation of

motor unit force and calculated IR, and may also explain the reduced compensatory capacity of remaining motor units in both human and mouse forms of ALS (Fig 2-9). We suggest that selective motor axon die-back in the $SOD1^{G93A}$ mice occurs by two parallel processes 1) preferential denervation of the fast-twitch, large type IIB muscle fibers and 2) the parallel conversion of remaining large fibers to smaller phenotypes. As the sprouting competence of the large caliber motor axons innervating the different type II fibers is very low (Frey et al., 2000), significant functional motor unit enlargement does not happen, and very few fibers are converted due to reinnervation by collateral axonal sprouting (Fig 2-9*C*). Instead, conversion occurs due to the increased recruitment of the remaining motor units, due to partial denervation and intrinsic hyperexcitability of the motoneurons (Kuo et al., 2004). As muscle fibers convert, we hypothesize that the sprouting competence of the motoneurons that innervate them increases, explaining the increased motor unit sizes seen in EMG (Kennel et al., 1996) and *in vivo* motoneuron visualization studies (Schaefer et al., 2005) on symptomatic $SOD^{G93A}$ mice. Coincident with this increase in IR there is also a reported reduction in the specific force of muscle fibers, so that motor unit force would become further disassociated from the IR.

As muscle biopsies are not routinely done on ALS patients, it remains to be seen whether preferential denervation and conversion cause changes in the muscle fiber phenotype proportions, These changes would account for the dissociation seen in human patients, where the forcefulness of motor units does not correspond to their size as measured by EMG (Milner-Brown et al.,

1974;Venkatesh et al., 1995;Vogt and Nix, 1997). Single muscle fiber data indicating no change in the specific force of muscle fibers from human patients indicates dissociation may result from the same mechanisms as we have suggested for the $SOD1^{G93A}$ mice (Krivickas et al., 2002). Our hypothesis for preferential motor unit loss will have to be slightly modified to accurately represent the disease progression in ALS patients for two reasons. First, in larger mammals like humans, the proportion of slow fibers in muscles increases; human gastrocnemius muscles are composed of 50% slow-twitch muscles (Edgerton et al., 1975). As the motor axons innervating these muscles are more competent at collateral axonal sprouting (Frey et al., 2000) the IR of the remaining motor units will increase. Secondly, the contractile properties of the motor units dictate differences in the IR, which increase in proportion to the size of the mammal (for example rats (Tötösy de Zepetnek et al., 1992) and cats (Rafuse et al., 1997)). Thus, the IR of slower, less forceful motor units will be much smaller than the more forceful motor units which are preferentially lost. The variation in the IR of motor units would mask the preferential enlargement of the smaller motor units. Mapping of motor endplates and axons in human ALS patients does indeed reveal that motoneurons exhibit a reduced ability to increase their IR, as compared to patients with other conditions of partial denervation (Coers et al., 1973).

Preferential loss of the most forceful, largest motor units will cause a loss of force and size of the motor units, in addition to causing a disassociation between motor unit size and force. In light of these findings, contractile force

recordings will have to be carried out during the lifespan of the $SOD1^{G93A}$ mouse in order to establish an accurate time-course of functional neuromuscular deficits. The characterization and enumeration of motor units will reveal whether functional deficits are apparent at the earliest sign of peripheral nerve die-back seen in the hindlimbs of $SOD1^{G93A}$mouse. We will also be able to elucidate whether motor units that have been converted to less forceful phenotypes gain the ability to sprout. Activity-dependent conversion and consequent changes to the compensatory ability of the neuromuscular system is of interest in the future rehabilitation efforts of ALS patients.

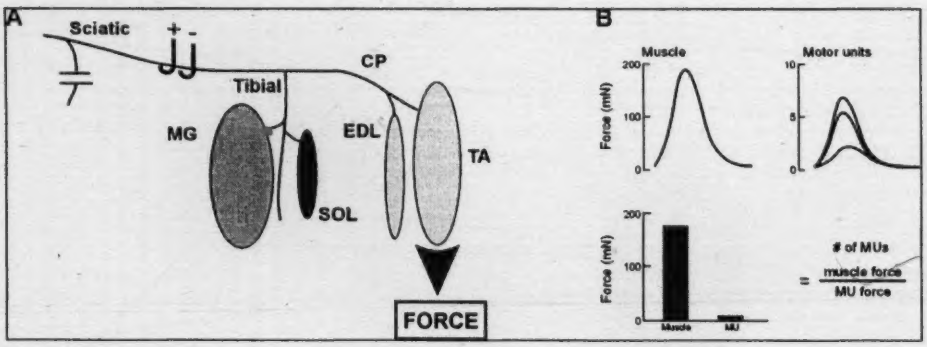


**Figure 2-1** Schematic illustration of the experimental set up for recording whole muscle and motor unit contractile force from mouse tibialis anterior (TA) or MG muscles. *A)* Supra-maximal stimulation of the sciatic nerve through silver wire electrodes elicits whole muscle contractile twitch and tetanic forces; stimulation of teased ventral root filaments resulted in all-or-none motor unit contractile force. As the amplitude of the stimulation to the teased ventral roots increases, motor units with higher thresholds are progressively recruited, resulting in the incremental increase of the recorded muscle contractile force *(B)*. Template subtraction was used to calculate the force of the recruited motor units, and the number of motor units was estimated by dividing the whole muscle twitch force by the average motor unit force.

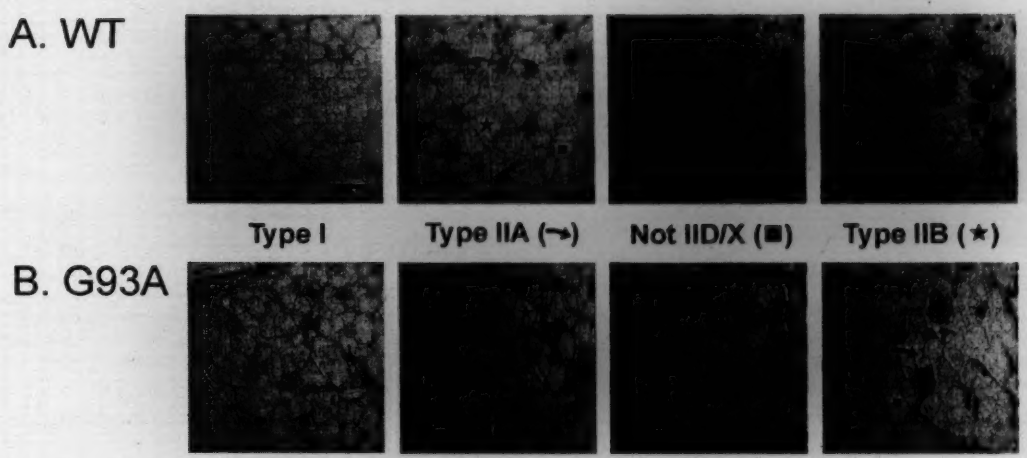


**Figure 2-2** Immunohistochemistry for Myosin Heavy Chain Isoforms types I, IIA, not IID/X and IIB in cross-sections of TA muscles of A) $SOD1^{WT}$ and B) $SOD1^{G93A}$ mice. The scale bar is 100μm.

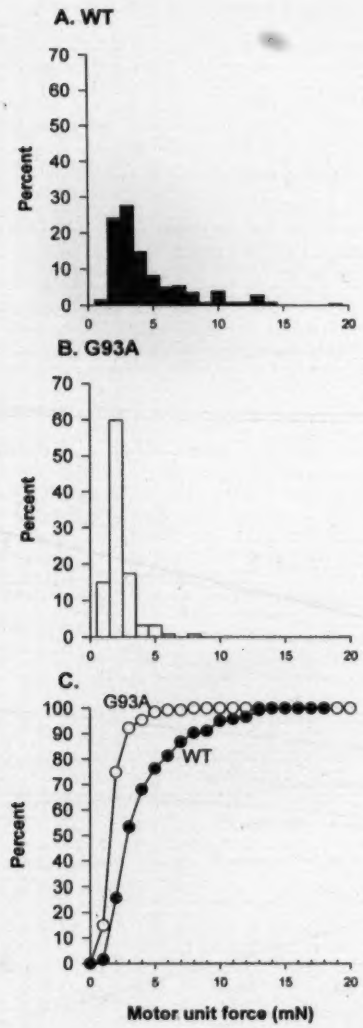


**Figure 2-3** Changes in the distribution of motor unit forces in the $SOD1^{G93A}$ mouse TA muscles, as compared to age matched $SOD1^{WT}$ control mouse TA muscles. *(A)* In the $SOD1^{WT}$ the distribution was skewed towards small motor units, with a range of motor units from a minimum of 0.5 to a maximum of 18.4mN. Approximately a third of all motor units produced forces above 4.0mN. *(B)* In the $SOD1^{G93A}$ the average motor unit force was half of that in the $SOD1^{WT}$ mouse TA muscles, and only a tenth of the motor units produced forces above 4.0mN; the maximum motor unit force was only 8.1mN. *(C)* The increase in the proportion of less forceful motor units is best illustrated by a cumulative frequency histogram. There was a leftward shift of motor unit forces, indicating that there was a loss of the most forceful motor units.

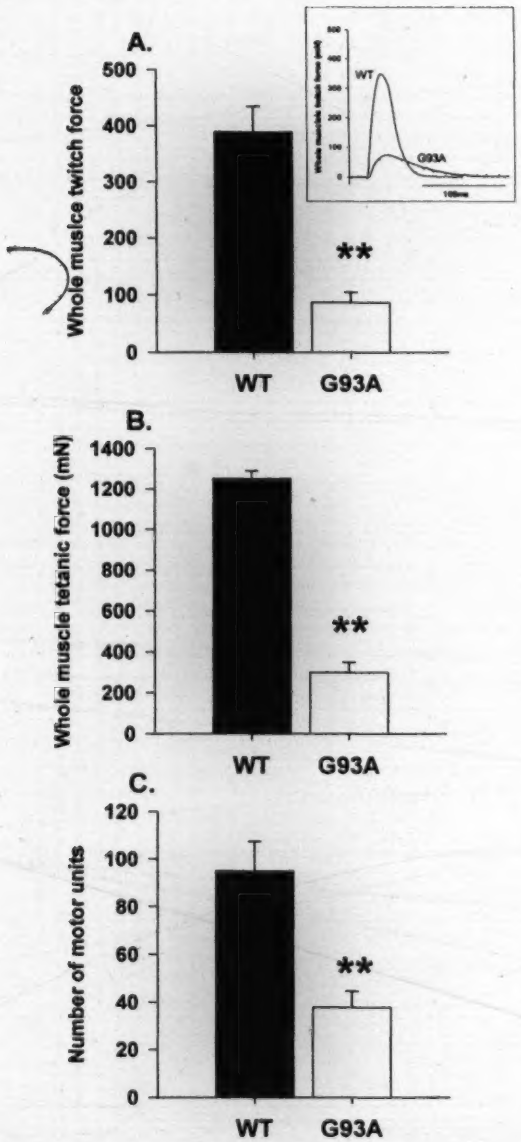


**Figure 2-4** There was a significant decline in the whole muscle twitch *(A)* and tetanic *(B)* contractile forces in the $SOD1^{G93A}$ mouse TA muscles, and a parallel loss of motor units, as compared to control $SOD1^{WT}$ muscles. *A)* The representative tracing of whole muscle twitch force indicates a decline in force, and a concomitant increase in the half-relaxation time of the muscle. The whole muscle twitch and tetanic forces decline in parallel *(A,B)* to approximately a fifth of the control $SOD1^{WT}$ mouse TA muscle values. *C)* In parallel with the decline in whole muscle force, there is also a significant loss of motor units from the $SOD1^{G93A}$ mouse TA muscle, as compared to age-matched controls; the number of motor units drops to ~50%. (Data are presented as mean $\pm$ SE and statistical significance is indicated as ** for $p<0.01$)

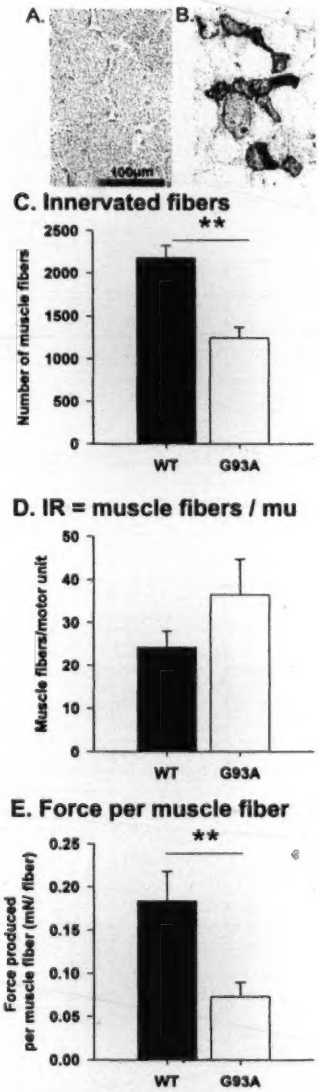


**Figure 2-5** A) Representative cross-sections of 60 day old $SOD1^{WT}$ and $SOD1^{G93A}$ mouse TA muscles. The sections were stained with neural cell adhesion molecule (NCAM). NCAM is expressed by muscle fibers when they are denervated, and the expression of NCAM is an earlier sign of denervation than deformation and atrophy. In the $SOD1^{WT}$ mouse TA muscle *(A)* the muscle fibers do not express NCAM, indicating that they are all normally innervated. In the $SOD1^{G93A}$ mouse TA muscles (*B)* there are muscle fibers which express NCAM. *(C)* The number of N-CAM negative muscle fibers that are innervated by motor axons, is reduced by approximately half in the TA muscles of the $SOD1^{G93A}$ mice as compared to the $SOD1^{WT}$ mice. *(D)* However, the average number of muscle fibers per motor unit (innervation ratio; IR) does not decline in the $SOD1^{G93A}$ mouse TA muscle, as compared to age-matched control. Despite the static IR, the force produced by each motor unit declines (Fig 2- 2), indicating that the contractile force producing capacity of each motor unit must be reduced *(E)*. (Data are presented as mean ± SE and statistical significance is indicated as ** for $p<0.01$ and * for $p<0.05$)

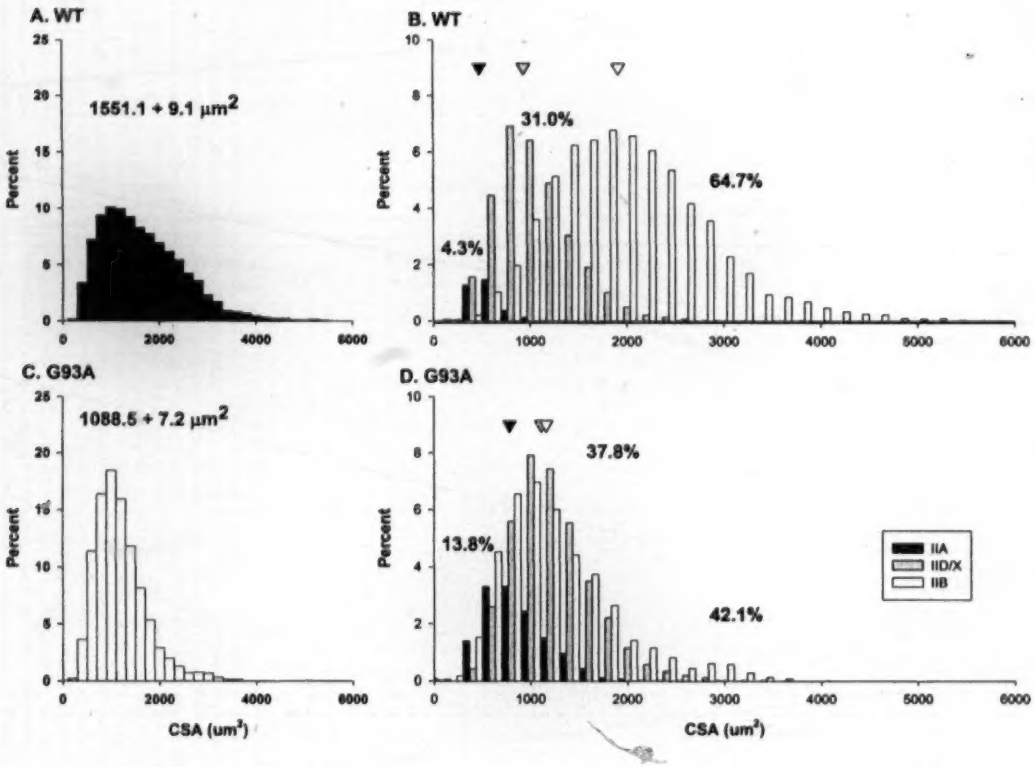


**Figure 2-6** Distribution of fiber cross-sectional area (CSA) in the $SOD1^{WT}$ and $SOD1^{G93A}$ mouse TA muscles. *(A)* In the $SOD1^{WT}$ mouse TA muscle, the distribution of fiber CSA was skewed towards the right, with a high proportion of muscle fibers having large CSA. *(B)* The skewed distribution was accounted for by the differences in the proportion of the different muscle fiber types; the greatest proportions of muscle fibers were the large type IIB's in the $SOD1^{WT}$ mouse TA muscle. There were smaller percentages of type IIA or IID/X muscle fibers, which were significantly smaller than the type IIB fibers. *(C)* The distribution of CSA in the $SOD1^{G93A}$ mouse TA muscle was also skewed, but the range of CSA and the average CSA are both smaller than in the age matched $SOD1^{WT}$ mice. *(D)* The different distribution was due to the change in the proportion of muscle fiber phenotypes, in that there was no longer a predominance of type IIB muscle fibers and also because the average CSA of the type IIB muscle fibers were smaller than in the $SOD1^{WT}$ mouse TA muscle. In addition to the decline in the average CSA of the largest muscle fibers, the smallest type IIA and IID/X muscle fibers hypertrophy resulting in a smaller difference between the CSA of the different muscle fiber phenotypes. Inverted triangles indicate the mean CSA values for each muscle fiber type.

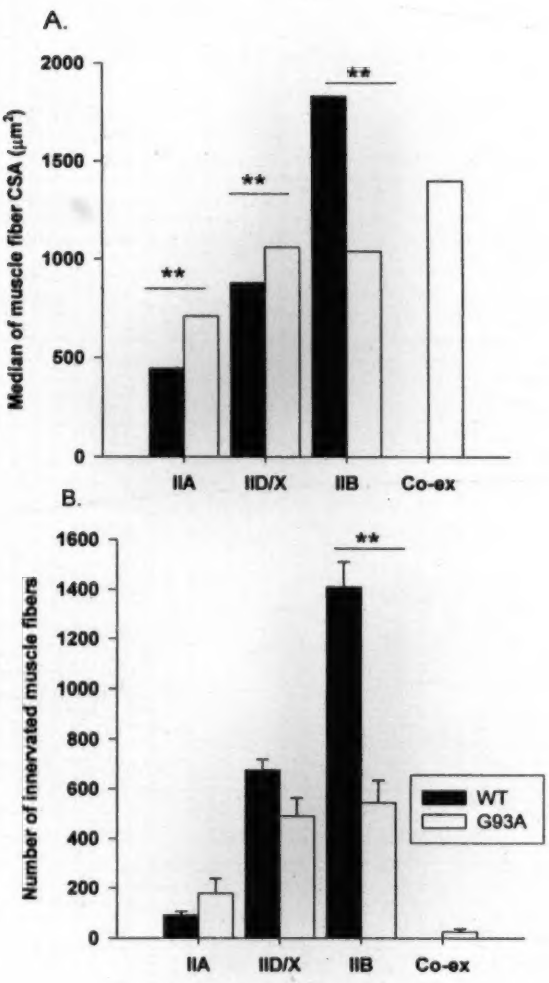


**Figure 2- 7** Changes in the size (*A*) and the number of innervated muscles fibers (*B*) which were classified on the basis of their MHC isoform immunohistochemistry. (*A*) The median of the IIB muscle fiber CSAs was significantly smaller in the $SOD1^{G93A}$ mouse TA muscles as compared to the $SOD1^{WT}$ TA muscles, while the type IIA and IID/X fibers were significantly larger (Significant differences determined with non-parametric tests as the distributions are not normal). (*B*) The number of type IIB fibers was reduced by ~50%, but there was no significant change in the number of type IIA or IID/X muscle fibers, as compared to the number of muscle fibers in the $SOD1^{WT}$ mouse TA muscle. There were no innervated muscle fibers that co-express different MHC isoforms in the $SOD1^{WT}$ TA muscle, while only a small proportion of muscle fibers co-expressed two different MHC isoforms in the $SOD1^{G93A}$ mouse TA muscle. (Data are presented as mean $\pm$ SE and statistical significance is indicated as ** for $p<0.01$ and * for $p<0.05$)

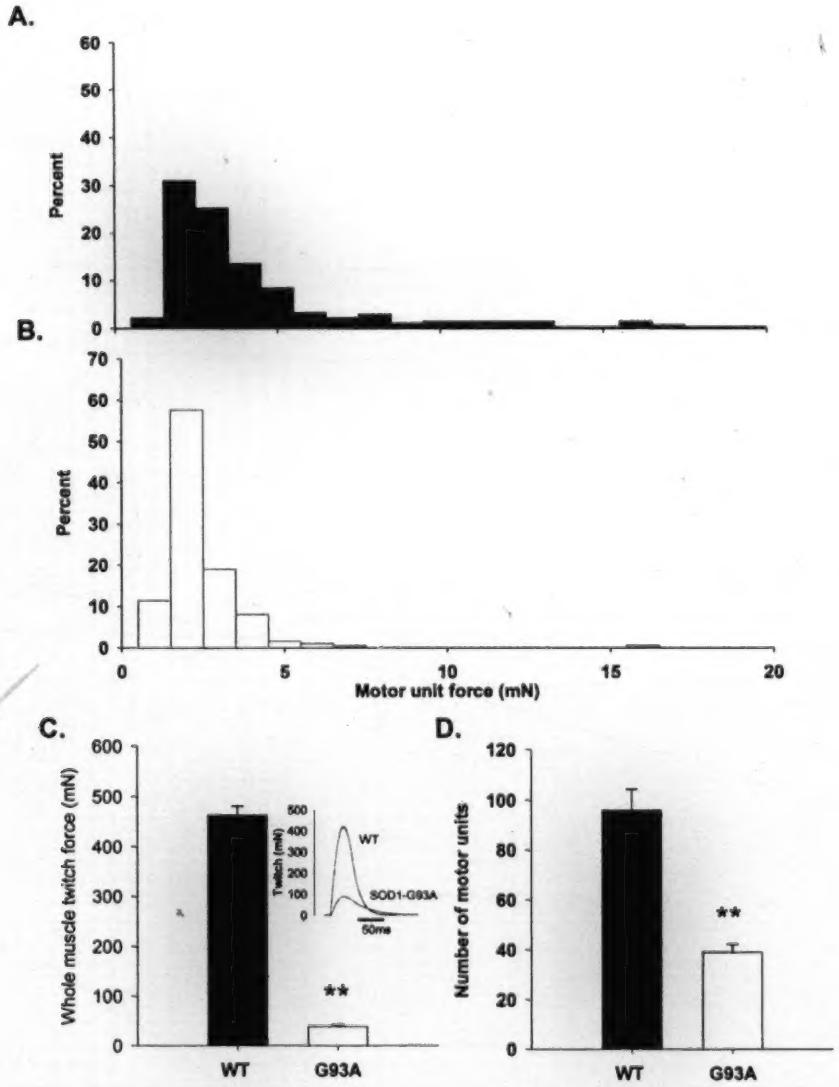


**Figure 2- 8** Motor unit force declined in MG muscles, in parallel with a decline in whole muscle twitch force and a loss of functional motor units. *(A)* In normal, 60-day old $SOD1^{WT}$ mouse MG muscles, the distribution of motor unit forces were skewed towards the left, with a high proportion of less forceful motor units. *(B)* The distribution of motor units in the $SOD1^{G93A}$ mouse MG muscle was also skewed to the left, but there was only motor unit that has a force over ~6mN. *(C)* In parallel with the loss of the most forceful motor units from the MG, the whole muscle twitch force declined by ~80%. A representative force tracing is seen in the inset. *(D)* We estimated that the number of intact motor units also declined in the 60-day old $SOD1^{G93A}$ mouse MG muscle, by ~60%, same as in the TA muscle.

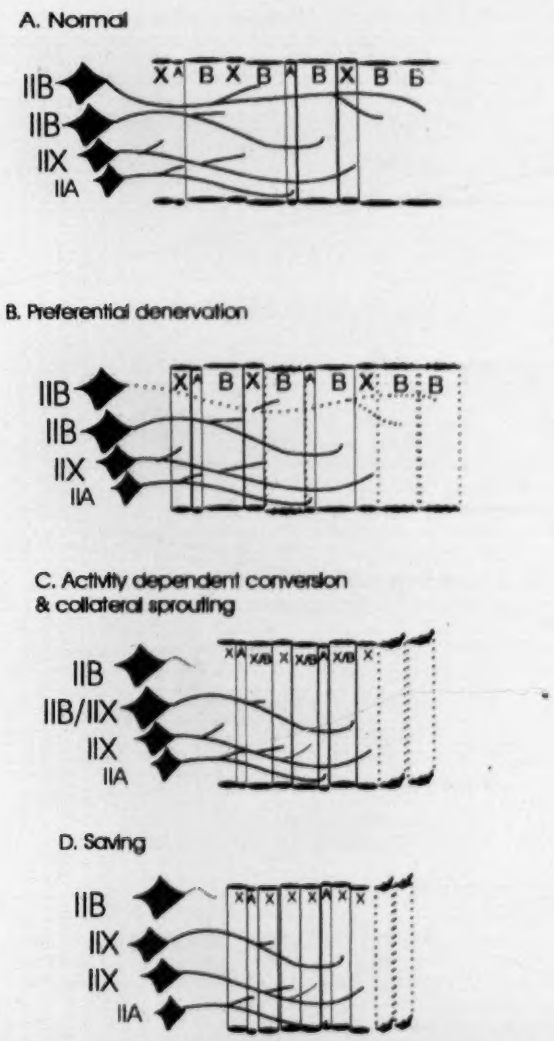


**Figure 2-9** A possible mechanism to describe neuromuscular change in pre-symptomatic SOD1$^{G93A}$ mouse TA muscles. *(A)* In the age-matched control SOD1$^{WT}$ mouse TA muscles, muscle fibers are innervated by intact motor axons, and there is no co-expression. *(B)* In early pre-symptomatic stages, there is withdrawal of axon branches preferentially from the largest motor units, which innervate type IIB muscle fibers. *(C)* Not all of the motoneurons that innervate type IIB fibers die-back, and those motor units remain intact must increase their activity to compensate for the selective and progressive loss. As a result of increased activity, the muscle fiber phenotype of the remaining muscle fibers changes to slower, more oxidative phenotypes. This conversion is first evidenced by a decrease in CSA. There is also some collateral axonal sprouting from smaller motor units that innervate type IIA and IID/X muscle fibers, resulting in co-expression of muscle fiber phenotypes. *(D)* Collateral sprouting and activity dependent conversion will result in co-expression of muscle fiber phenotypes, and a decrease in CSA of the innervated muscle fibers. Denervated muscle fibers that are not reinnervated become angulated and atrophy. Eventually, the proportion of type IIB muscle fibers becomes very low. As the type IIB muscle fibers produce the greatest amounts of force, the average force producing capacity of the remaining muscle fibers is reduced.

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# Chapter 3

# TIME COURSE OF PREFERENTIAL MOTOR UNIT LOSS IN THE SOD1$^{G93A}$ MOUSE MODEL OF AMYOTROPIC LATERAL SCLEROSIS (ALS)

## 3.1 INTRODUCTION

The neurodegenerative disease amyotrophic lateral sclerosis (ALS) is characterized by progressive death of motoneurons and consequent skeletal muscle denervation, manifesting as weakness and eventually paralysis. Approximately 10% of ALS cases are familial (fALS), and of these 20% have been linked to mutations of the superoxide dismutase (SOD) gene (Rosen et al., 1993). In the most widely used model of fALS, human mutant cytosolic Cu/Zn SOD1 with a glycine to alanine conversion at the 93$^{rd}$ codon is over-expressed in transgenic mice (SOD1$^{G93A}$ mice; (Gurney et al., 1994).

SOD1$^{G93A}$ mice develop an ALS-like disease with the initial symptoms including fine tremors and weakness developing in the hind-limbs at ~90 days of age, followed by severe hind-limb paralysis at ~120 days of age (Gurney et al., 1994). Critical motoneuron loss from the lumbar spinal cord is coincident with the onset of symptoms (Chiu et al., 1995) but peripheral dysfunction is evident in pre-symptomatic mice (Fischer et al., 2004). In agreement with the anatomical evidence of preferential die-back of the largest axons (Fischer et al., 2004) the most forceful motor units are lost from hindlimb muscles in presymptomatic mice (Chapter 2) with concomitant denervation of the fast-twitch muscle fibers (Frey et

al., 2000). Anatomical and electrophysiological evidence from human ALS studies also indicates that the less forceful, slower motor units preferentially survive in ALS (Dengler et al., 1990). Here we explore the issue of preferential survival by comparing the time course and extent of motor unit loss in both fast- and slow-twitch muscles in the $SOD1^{G93A}$ mouse model of ALS.

Previously, electromyographic (EMG) recordings have been used to study the time-course of neuromuscular deficits in $SOD1^{G93A}$ mouse muscles with varying results. Using needle EMG the earliest significant decrement in whole compound muscle action potential (CMAP) and motor unit loss has been reported in the gastrocnemius muscle at 47 days of age, after which the whole muscle potential continued to decline linearly until end-stage disease (Kennel et al., 1996). However, using the same method, Azzouz et al., (1997) detected a biphasic decline of gastrocnemius CMAP and motor unit number, with a small, slow decline between 60 and 100 days of age. The only study to include slow-twitch muscles used surface EMG electrodes to record from all of the hind-limb muscles simultaneously; Shefner and colleagues (1999) found that CMAP begin to rapidly decline at ~60 days of age, concomitant with a loss of functional motor units at the same time (Shefner et al., 1999;Shefner et al., 2001).

To reconcile the findings of the different studies examining the time course of motor unit loss we measured contractile force and enumerated motor units from four morphologically distinct hindlimb muscles throughout the lifespan of $SOD1^{G93A}$ mice. Motor unit loss was significant from fast- but not slow- twitch muscles at 40 days of age, 50 days prior to the reported onset of overt

symptoms. No deficits in whole muscle contractile force or motor unit numbers were seen in the slow twitch soleus (SOL) muscle until 90 days of age, coincident with symptom onset. The loss of motor units from the fast-twitch hindlimb muscles was initially quick, and then plateaued in the symptomatic phase of the disease. Aside from establishing that motor unit loss is biphasic, this is the earliest report of functional motor unit loss in the $SOD1^{G93A}$ mouse model of ALS. Our findings established that muscle fiber type composition rather than function determines preferential vulnerability of motor units.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Generation of SOD1$^{G93A}$ mice

Transgenic male mice expressing a high copy number of the human SOD gene with a glycine to alanine base pair mutation at the 93$^{rd}$ codon of the cytosolic Cu/Zn superoxide dismutase (SOD1) gene (SOD1$^{G93A}$; B6JSL-TgN (SOD1-G93A) or a high copy number of normal human SOD1 gene (SOD1$^{WT}$;B6JSL-TgN (SOD1) were obtained from Jackson Laboratories, USA. The transgenic male SOD1$^{G93A}$ and SOD1$^{WT}$ mice were bred to non-transgenic B6JSL hybrid females, and resulting progeny was identified using standard PCR protocol for the human SOD1 (Rosen et al., 1993) performed on ear biopsy samples taken at the time of weaning (approximately 21 days of age). The mice were identified using ear punches, and kept in standard animal housing with free access to food and standard rodent chow. The University of Alberta Health Sciences Laboratory Animal Ethics committee approved all experimental procedures, which were in accordance with the Canadian Council for Animal Care.

To avoid ambiguity associated with reported gender-related differences in mean survival time of SOD1$^{G93A}$ mice (Veldink et al., 2003), we used only male mice in the present study. The male SOD1$^{G93A}$ mice became symptomatic at approximately 90 days of age. Symptoms include fine shaking, tremors and spasticity in the hind-legs (Chiu et al., 1995). Complete paralysis of the hindlimbs occurs within 30 to 40 days after the onset of the symptoms. The

$SOD1^{WT}$ mice have not been reported to develop ALS-like disease (Gurney et al., 1994).

Four morphologically distinct hind-limb muscles were examined: the fast-twitch tibialis anterior (TA), the extensor digitorum longus (EDL) and medial gastrocnemius (MG) muscles, and the slow-twitch soleus (SOL) muscle. We enumerated motor units and recorded maximal isometric contractile forces from male $SOD1^{G93A}$ mouse muscles at 10 day intervals. The number of muscles that were recorded from at each age group is summarized in Table 3-1.

### 3.2.2 Electrophysiological studies

*Surgery* Mice were anaesthetized with an intra-peritoneal (IP) injection of a cocktail made up of Ketamine (100mg/mL), Atravet (10 mg/mL) and 0.9% sodium chloride, at a dosage of 17.5mL/kg body weight. At regular time intervals additional anaesthetic was administered IP to maintain anaesthesia. The body temperature of the mice was maintained using a heat lamp. The TA, EDL, MG and SOL muscle tendons were isolated and individually attached with 4.0 silk to a strain gauge (Kulite model KH-102) to record the isometric force produced by each muscle in response to stimulation of the sciatic nerve (Fig 3-1*A*). Tendons of the plantaris and lateral gastrocnemius muscles were cut. Two silver wire electrodes were placed alongside the sciatic nerve and a controlled stimulation was applied to the nerve to evoke unitary and maximal isometric twitch and tetanic isometric contractions. The electrodes were sutured into place to prevent dislocation during stimulation. Hindlimbs of anaesthetized mice were immobilized

by clamping the knees and the ankles, while being careful not to interfere with the blood supply to the muscles.

*Recordings* Evoked isometric forces were amplified and visualized on an oscilloscope and digitized using Axoscope (Version 8.0, Axon Instruments, USA). Muscle length was adjusted for maximal isometric twitch force in response to stimulation of the sciatic nerve. Whole muscle twitch and tetanic forces were recorded in response to single and repetitive suprathreshold (twice threshold amplitude) stimulation of the sciatic nerve at 0.5 and 100Hz, respectively. The tetani lasted for 210 ms and the maximum tetanic force was measured. Force recorded from TA muscle is shown in the example of a 60-day control mouse (Fig 3-1B)

To estimate the number of motor units in hind-limb muscles of the mice, we used a modified version of the mechanical motor unit number estimation (MUNE) that was first described by McComas and colleagues in 1971. The mechanical MUNE method, which records up to 10 incremental steps in the M=wave makes the important assumption that each distinguishable incremental increase represents the recruitment of an additional motor unit. However, the method fails to account for the alternation of motor units, namely that motor units can be recruited in numerous combinations by stimulation of a motor nerve (Galea et al., 1991). Alternation results in overestimation of motor unit numbers, and increases progressively with the number of motor units recruited (Stein and Yang, 1990). In this study we attempted to overcome the problem of alternation by not counting the incremental increases in muscle twitch force, but instead

calculating the average of 8 to 15 randomly chosen motor unit forces associated with incremental force increases at different stimulus amplitudes (Fig 3-1*B*).

Beginning at a stimulus voltage of 100μs duration that was below the threshold for recruitment of motor units, we applied a manually controlled graded 0 to 10V electrical stimulus to the sciatic nerve at a frequency of 0.5 Hz. At each stimulus intensity the few motor units with thresholds very close to that voltage were variably recruited (due to alternation); the stimulus voltage was held constant for several pulses so that the change in isometric twitch force resulting from the intermittent firing of motor units with similar thresholds could be recorded. Thus, every possible combination of motor unit addition was recorded. The voltage was manually raised until the voltage required to elicit the maximal isometric twitch force was reached. Even though the motor units added in multiple combinations at each of the stimulus intensities, we can still assume that each distinguishable "all-or-none" change in the isometric force is the addition of individual motor units, as in the incremental method of McComas (1971). However, in contrast with the incremental method, we cannot count the number of incremental steps to yield motor unit number, as we know that we have intermittent firing and associated alternation.

We calculated the force associated with 8 to 15 random increments in whole muscle force chosen by the computer from various stimulus voltage intensities. Average motor unit force was obtained by overlaying the digitized forces in MATLAB and template subtraction of the forces to give the individual motor unit twitch forces. As motor units were chosen at random a representative

sample of motor unit forces was obtained. In addition, if the threshold of the chosen motor units is different enough, we can assume that they are not the result of alternation. The average motor unit force was divided into the maximal isometric twitch force to give an estimate of the number of motor units, as previously described (McComas, 1995; Fig 3- 1B).

### 3.2.3 Myosin heavy chain determination

We used electrophoresis to quantitatively determine the MHC-isoform for the TA, EDL, MG and SOL muscles, as previously described (Hämäläinen and Pette, 1993;Martins et al., 2006). In brief, muscles were homogenized in a buffer containing 100 mM $Na_4P_2O_7$ (pH 8.5), 5 mM EGTA, 5 mM $MgCl_2$, 0.3 M KCl, 10 mM DTT and 5 mg/ml of Complete™ protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and stirred for 30 minutes on ice. Homogenates were then cleared by centrifugation (12,000×g at 4°C), diluted 1:1 with glycerol and stored at –20 °C. Prior to dilution in Laemmli-lysis buffer and boiling for five minutes, the protein concentration of the samples was determined (Lowry et al., 1951) Following the dilution, 1 μg of total protein was loaded into each lane of a 4% polyacrylamide stacking gel that also contained glycerol. Under a constant voltage of 275 volts, the samples were separated under denaturing conditions on a 7% polyacrylamide separating gel that also contained glycerol, for 24 hours at 8°C (Hoefer SE600;Amersham Biosciences). Gels were silver stained for the MHC isoforms, as previously described (Oakley et al.,

1980). The silver stained gels were then evaluated in triplicate by densitometry (Syngene).

### 3.2.3 Statistical analysis

Data are presented as means ± standard errors (SE). Statistical significance between experimental groups was assessed Students' t-test in Sigma Plot (SPSS, Version 8.0). Differences were considered statistical significant if $p<0.05$. In the Figures significant is denoted by asterixes, with * for $p<0.0.5$ and **for $P<0.03$. Power calculations were made to confirm sufficient power and/or sample size using PC-Size (STATOOLS, Version 2.13, 1986). Power values of at least 0.8 were required to consider results significant.

## 3.3 RESULTS

### 3.3.1 Comparison of $SOD1^{WT}$ and non-transgenic control mice

$SOD1^{WT}$ transgenic mice, which express normal human SOD1, do not develop symptoms of weakness or paralysis during the life-span of the $SOD1^{G93A}$ or even at even at 1 year of age (Chiu et al., 1995;Gurney et al., 1994). Before we used the $SOD1^{WT}$ mice as controls, we measured contractile isometric force and determined the number of motor units in the hindlimbs of both $SOD1^{WT}$ (n=7) and control, non-transgenic mice (n=4). Neither the number of motor units (Fig 3-2*A*), nor the whole muscle contractile twitch force (Fig 3-2*B*) differed in any of the hindlimb muscles when the $SOD1^{WT}$ and age-matched non-transgenic mice were compared at 120 days of age using a Student's t-test. Hence, we assumed that the contractile force and motor unit numbers for the $SOD1^{WT}$ and age-matched non-transgenic mice do not differ at any time point throughout the life-span of the $SOD1^{G93A}$ mouse. For this paper, we have combined our data from $SOD1^{WT}$ mice and non-transgenic mice, hereafter referred to as the control group (CON, n=11 at 120 days of age, n= 4 at 80 days of age and n=4 at 40 days of age).

### 3.3.2 Phenotypic composition of four hindlimb muscles

Before determining the time course of motor unit loss in the four hind-limb muscles of the $SOD1^{G93A}$ and control mice, we established muscle fiber type composition in the muscles of the $SOD1^{WT}$ mice using gel electrophoresis tp separate and quantify the various MHC isoforms. Previously, the fast-twitch

antagonistic TA and MG muscles have been shown to lose functional motor units before the onset of overt symptoms in the hindlimbs of SOD1$^{G93A}$ using both EMG (Azzouz et al., 1997;Kennel et al., 1996) and contractile force measurements (Chapter 2). Both the TA and MG muscles are composed of ~90% type II muscle fibers (Fig 3-3*A*). However, the distribution of the different type II fibers differs somewhat between the TA and MG muscles (Fig 3-3*B,C*). In the TA muscle, the proportion of type IID/X fibers was ~32%, as compared to ~15-20% in the MG muscle and the percentage of type IIB fibers was also significantly lower (Fig 3-3*C*). When we compared the number of motor units in the 60-day old SOD1$^{G93A}$ mice, the magnitude of motor unit loss in the TA and MG was the same (Chapter 2).

To determine whether an increased proportion of type I fibers confers increased protection to muscles in the SOD1$^{G93A}$ mouse model, a slow-twitch hindlimb muscle was included in the study. The only previous study to compare whole muscle contractile forces of a fast- and slow- muscles in the SOD1$^{G93A}$ mouse found a significant decline in the whole muscle tetanic force at 120-days for the EDL, but not the SOL muscle (Derave et al., 2003). The ankle extensor slow-twitch SOL muscle contained 56% fast MHC II isoforms and 44% slow MHC-I isoforms (Fig 3-3*D*). In contrast, the EDL muscle contains ~96% fast MHC II isoforms, including isoforms IIa, IId/x and IIb, and only 4% slow MHC-I isoforms (Fig 3-3*F*). The muscle fiber type proportions found here in all the CON mice muscles agrees well with previously published mouse data (Hämäläinen and Pette, 1993).

### 3.3.3 Selective muscle susceptibility during the pre-clinical phase of disease in the SOD1$^{G93A}$ mice

Tetanic force produced by the SOD1$^{G93A}$ mouse EDL was already significantly decreased as compared to the control mouse EDL at 80 days of age (Fig 3-4*A*). The mean tetanic force declined by approximately half (44.6%) in the EDL while that produced by the SOL muscle was not significantly different between the age-matched control and SOD1$^{G93A}$ mice (Fig 3-4*A*). This is the first report of a selective susceptibility of fast-twitch whole muscle contractile force, as compared to slow-twitch muscle contractile force before the onset of overt symptoms in the SOD1$^{G93A}$ mouse.

To further investigate the selective neuromuscular deficit in the fast-twitch EDL but not the slow-twitch SOL muscles in the pre-symptomatic SOD1$^{G93A}$ mouse, we next determined the number of intact motor units in these muscles. Motor unit force was measured by incremental stimulation of the sciatic nerve to evoke all-or-none increments in twitch force (see methods and Fig 3-1). A mean motor unit force was obtained from 8-15 motor units, allowing us to calculate the number of functional motor units. At 80 days of age, there was a decline of 47.5% in the number of EDL motor units in the SOD1$^{G93A}$ mice, as compared to control mice. In contrast, there was no significant loss of motor units in the SOL muscle at the same age (Fig 3-4*B*).

The magnitude of the decline in the mean tetanic force produced by the EDL muscle in the SOD1$^{G93A}$ mice was the same as the decline in the number of

functional motor units in the EDL muscle (Fig 3-4). The parallel decline in whole muscle force and motor unit number indicates that there was little or no functional enlargement of the remaining motor units in the EDL muscles. In healthy mammalian muscle there would be an increase in average motor unit force to compensate for partial denervation (Gordon et al., 1993;Rafuse et al., 1997). The lack of functional enlargement may also be indicative of a reduction in either the cross-sectional area (CSA) or the specific force of the reinnervated muscle fibers. However previous authors have failed to find such changes in pre-symptomatic fast- and slow-twitch muscles (Atkin et al., 2005). The early uncompensated motor unit loss in the EDL muscle is surprising, as there is no readily detectable behavioural changes at 80-days of age (Weydt et al., 2003). It does, however, agree with our earlier findings that motor unit force declined in parallel with a loss of motor units in the MG and TA muscles at 60-days of age (Chapter 2). It is possible that the behavioural tests of hindlimb function are not sensitive enough to detect subtle changes in force production in one or more fast-twitch hindlimb muscles.

### 3.3.4 Selective motor unit loss at earliest signs of disease pathology

We next wanted to determine how early we could detect the loss of functional motor units. In the $SOD1^{G93A}$ mouse the earliest signs of pathology at 30–37 days of age include aggregation of mutant SOD1 proteins in muscle (Turner et al., 2003) and motoneurons (Johnston et al., 2000) and vacuole formation in motoneurons and their axons (Chiu et al., 1995). We hypothesized

that the early pathology seen in the hind-limbs muscles and peripheral nerves of the SOD1$^{G93A}$ mouse would result in early functional motor unit loss.

As expected from the normal number of motor units in the 80-day old SOD1$^{G93A}$ SOL muscle (Fig 3-4), the number of motor units in the 40-day old SOD1$^{G93A}$ SOL muscle was not significantly different from the number of motor units in the control SOL (Fig 3-5*A*). In contrast, the number of motor units in the fast-twitch MG muscle was significantly reduced by ~22% as compared to control mice at 40-days of age (Fig 3-5*B*). There was also a trend for the number of motor units to decrease in the fast-twitch EDL and TA muscles (Fig 3-5). This early loss at 40-days of age, occurs well before other investigators reported denervation with electromyographic (Azzouz et al., 1997;Kennel et al., 1996;Shefner et al., 2001) or immunohistochemical methods (Frey et al., 2000). This early and selective pattern of motor unit loss from the fast-twitch muscles indicates the increased susceptibility of the larger motoneurons which innervate the fast type IIB and IID/X muscle fibers. Motoneurons that innervate the type I and IIA muscle fibers, as in the SOL muscle, are smaller and appear to be less susceptible in pre-symptomatic SOD1$^{G93A}$ mice.

### 3.3.5 Muscle phenotype dictates susceptibility to motor unit loss

To determine whether muscle fiber type proportions dictate motor unit susceptibility throughout the entire life-span of the SOD1$^{G93A}$ mouse, we determined the number of motor units in the TA, MG, EDL and SOL muscles at 10 day intervals throughout the life-span of the SOD1$^{G93A}$ mouse.

In the slow-twitch SOL muscle there was no pre-symptomatic loss of motor units in the SOD1$^{G93A}$ mice as compared to age-matched controls. At 90 days of age, coincident with the onset of detected hind-limb weakness in the SOD1$^{G93A}$ mouse, there was a significant decline of ~20% in the number of motor units in the SOL muscle. The motor unit decline the SOL muscle continued until 100 days of age, by which time the mice were spastic and trembled (Chiu et al., 1995). From 100 to 120 days of age, the number of motor units remained constant at ~20 motor units, which is approximately 60% of the motor units in the control mouse SOL (Fig 3-6*A*). In contrast, the number of motor units in the EDL declined exponentially, with a sharp initial drop in motor unit numbers that was already significant at 40 days of age (Fig 3-6*A*). The sharp decline of motor units in the EDL muscle continued until 90 days of age, when the number of motor units reached a plateau of ~15 motor units, or ~40% of the motor units in the control EDL muscle (Fig 3-6*A*). There was no decline in the number of EDL motor units following the onset of symptoms of weakness and tremors in the SOD1$^{G93A}$ mouse at 90 days of age.

The MG and TA muscles, which also contained low proportions of slow MHC isoform expressing muscle fibers like the EDL, also exhibited an early and

exponential decline of motor units. The sharpest decline in motor unit numbers occurred in the TA muscle between 40 and 50 days of age. After 50 days of age the decline in TA motor units proceeded in a more gradual manner, until the motor unit numbers reached an initial plateau of 70 days of age. Thereafter, there was a second sharp drop in the number of TA motor units between 80 and 100 days of age, around the time of symptom-onset. There was no decline in the number of motor units in the TA after 100 days of age in the $SOD1^{G93A}$; ~20 TA muscle motor units remained until 120-days of age. The MG muscle had a lower proportion of type IID/X muscle fibers than the TA muscle, and did not exhibit as great an exponential decline of motor units as the TA muscle. The decline in MG motor units was biphasic, as in the TA muscle, with an initial decline from 40 to 60 days of age and a second significant decline from 80 to 100 days of age. This second phase of decline was followed by a plateau in motor unit numbers from 100 to 120 days of age, wherein only ~25 motor units remained in the MG muscle.

In all four hind-limbs, regardless of muscle fiber type composition or muscle function, there was no significant motor unit loss from 100- to 120-days of age (Fig 3-6*C*). As compared to the number of motor units in control mice, there were ~70, 45, 30 and 30% motor units remaining in the SOL, EDL, MG and TA muscles at 120-days of age, respectively. The high percentage of surviving motor units at 120-days of age in the SOL is due to the greater proportion of slow fiber types. This correspondence of surviving motor units to the proportion of

slower muscle fiber MHC isoforms provides further support for the idea that there is a preferential loss of motoneurons innervating the fast-twitch muscle fibers.

### 3.3.6 Contractile force declines in parallel with motor unit numbers

We have already shown that in the 80 day old SOD1$^{G93A}$ mouse EDL muscle, the loss of motor units resulted in a corresponding decrease in the force producing capacity of the fast-twitch EDL muscle (Fig 3-4). This correspondence between motor unit loss and decline in force producing capability indicates that there is no functional compensation for the loss of motor units in the EDL muscle at 80-days of age. It is possible that this uncompensated neuromuscular deficit in the EDL muscle is not detectable in behavioural tests because the EDL produces only a fraction of the force produced by the antagonistic MG and the agonist TA muscles.

Therefore, we wanted to determine whether there was any evidence of functional compensation in the fast-twitch MG and TA muscles during the life-span of the SOD1$^{G93A}$ mouse. We recorded muscle tetanic forces from the MG and TA throughout the life-span of the mouse and compared the magnitude and time-course of the fall in contractile force to the decline in motor unit numbers. In both the MG and TA muscles the contractile muscle forces decreased with age in the SOD1$^{G93A}$ mice as compared to age matched control mice, in which the tetanic forces remained constant (Fig 3-7*A,B*). In the SOD1$^{G93A}$ mouse at 40 days of age, there was already a significant decline in the force generating capacity of the fast-twitch TA and MG muscles. In both muscles the decline in

tetanic force is exponential, with a sharp initial drop that plateaus at ~100-days of age (Fig 3-7*A,B*).

In the fast-twitch TA and MG muscles the motor unit numbers fall in proportion to the decline in tetanic force (Fig 3-7*C,D*). Had there been compensatory axonal sprouting, we would have seen sustained twitch and tetanic isometric forces, despite a decline in motor unit numbers, a 5-fold increase in motor unit size compensating for loss of up to 85% of the motor units (Gordon et al., 1993). The parallel decline demonstrates that there is little or no functional compensatory enlargement of the remaining motor units in the $SOD1^{G93A}$ mice in the fast-twitch muscles studied.

## 3.4 DISCUSSION

The first major finding of our study was that there is an early and selective decline in the number of motor units in fast-twitch hind-limb muscles that was significant at 40-days of age, 50-days before the onset of symptoms in the SOD1$^{G93A}$ mouse model of ALS. This preferential loss of motor units was dictated by muscle fiber type proportions and not by muscle function. Our findings of selective motor unit vulnerability support immunohistochemical evidence indicating that denervation proceeds in a muscle-specific manner, with the denervation of type IIB muscle fibers before slower, type I, IIA and IID/X fibers in the triceps surae (Frey et al., 2000) and TA muscles of the SOD1$^{G93A}$ mouse (Chapter 2). Longitudinal visualization of motor units in SOD1$^{G93A}$ mice also revealed that only selective axons are maintained, and these are selectively able to compensate for the loss of other, degenerating axons (Schaefer et al., 2005). Although they did not draw parallels between slow and fast- motor units, the results of Schaefer et al. (2005) showed a greater number of innervated endplates in the slow-twitch SOL muscles, as compared to the fast-twtich EDL muscle.

With accumulating anatomical evidence for a die-back process in the SOD1 mouse (Fischer et al., 2004), it has become increasingly important to reconcile electrophysiological studies reporting contrasting rates of motor unit loss in pre-symptomatic SOD1$^{G93A}$ mice. Our present findings resolve the apparent differences in the time course of motor unit loss reported by the EMG studies of Azzouz et al (1997), Kennel et al (1996) and Shefner et al (1999,

2001). Although we agree with Kennel et al (1996), that motor unit loss from the gastrocnemius is already significant at 47-days of age, they unfortunately did not characterize the time course of motor unit loss and we are unable to compare our results. In contrast Azzouz et al (1997) reported that there was a dramatic decline in the number of motor units in the MG only after 100 days of age and upon this evidence suggested that motor unit loss was coincident with motoneuron death in the lumbar spinal cord in symptomatic $SOD1^{G93A}$ mice. We started our investigation earlier than any of the previous studies (40 days of age) and detected the biphasic decline in MG motor units. Indeed, the number of motor units in the MG does not undergo a striking change between 50 and 70 days of age when Azzouz et al (1997) began their investigation, leading them to find only one dramatic decline in motor unit number after 90-days of age.

Using two different methods to enumerate motor units from functional EMG recordings, Shefner et al (2002) consistently estimated the number of motor units in all the extensor and flexor muscles of the lower hindlimb. They reported an exponential loss of motor units, that is significant by ~60-days of age when they began their examination (Shefner et al., 1999;Shefner et al., 2002). However, they studied both fast- and slow-twitch muscles simultaneously, and neither the biphasic motor unit loss in the MG and TA muscles, nor the preferential loss of the fastest motor units could be detected. In order to observe the selective vulnerability of motor units, fast-twitch muscles had to be studied in isolation, and contrasted to slow-twitch SOL muscles as we have done here.

Our second major finding was that this selective vulnerability of the motor units innervating fast-twitch muscles was maintained throughout the life-span of the SOD1$^{G93A}$ mouse (Fig 3-6). In the fast-twitch muscles, there was an exponential decline in motor units, with a significant loss detected at 40 days of age. In the slow-twitch SOL muscle, there was no significant decline in the number of motor units until 90 days of age. The decline in motor unit numbers reached a plateau in all of the muscles we studied at ~100 days of age. In the SOL muscle, ~70% of the motor units remained at end-stage disease, corresponding to the proportion of muscle fibers that express slower MHC I and MHC IIA isoforms (~70%). This correspondence between muscle fiber type proportions and motor unit loss is supporting evidence for the selective vulnerability of motor units innervating the fast-twitch muscles.

In all four hind-limb muscles, regardless of muscle fiber type proportions, the loss of motor units proceeded in two stages; an initial rapid decline that reached a plateau in the symptomatic stage of the disease (Fig 3-6). This mirrors the disease progression in the ALS patients, where the fastest rate of motor unit loss is within the first year of disease diagnosis; after the first year, the loss becomes more gradual (Dantes and McComas, 1991). The selective vulnerability of motor units innervating fast-twitch muscles in the SOD1$^{G93A}$ mice also corroborates with findings in human patients: Dengler et al (1990) reported an increased susceptibility of motor units with larger twitch forces.

Selective loss of functional motor units may be linked to characteristics that distinguish motoneurons innervating muscle fibers expressing slow type I

MHC isoforms from those expressing fast type II MHC isoforms. According to the Henneman size principle, motoneurons that innervate fast motor units are those with the largest innervation ratios, soma sizes and axon calibers (Gordon et al., 2004;Henneman, 1985). Smaller motoneurons, which are recruited first under physiological conditions, innervate the smallest and slowest motor units. It has been shown that axons are susceptible in a caliber-specific order, with the largest caliber axons being the most susceptible to loss in mouse models of ALS (Bendotti et al., 2001;Fischer et al., 2004;Kong and Xu, 1998) and in human patients (Feinberg et al., 1999). Interestingly, when axons are converted to smaller calibers by experimentally inducing peripheral nerve injury and axon regeneration, they become less susceptible (Kong and Xu, 1999). Here, we provide further supporting evidence that motor unit type, and the associated differences in innervation ratio, axon caliber and soma size appear to have a role in specific vulnerability of motoneurons. Hence, larger soma size and axon diameter appear to be important determinants of differential motoneuron susceptibility and our results corroborate the hypothesis that implicates soma size and metabolic demands in the preferential susceptibility of motoneurons (Shaw and Eggett, 2000).

Our third major finding was that motor unit loss was paralleled by a decline in the force generating capacity of the hindlimb muscles in the SOD1$^{G93A}$ mouse. At 80-days of age, there was no evidence of functional compensation for the selective motor unit loss in the EDL (Fig 3-4). When we compared the decline in motor unit numbers to the drop in force generating capacity in the MG and TA

muscles throughout the life-span of the SOD1$^{G93A}$ mouse, we also found no evidence of functional compensation (Fig 3-7). Previous immunohistochemical work by Frey et al (2000) showed that the motoneurons innervating muscle fibers expressing slow MHC isoforms exhibit a compensatory sprouting response. Using transgenic mice that express both green fluorescent protein and mutant G93A SOD1, Schaeffer et al (2005) also showed a robust compensatory sprouting response in axons that were preferentially spared. Our results suggest that this sprouting process is ineffective and few, if any, functional connections are formed as a result of compensatory axonal sprouting. Work by Shefner (2001) that showed a proportional decline in CMAP and motor unit numbers is also indicative of a lack of compensatory motor unit enlargement.

Despite the early and significant fast-twitch muscle deficits, commonly employed behavioural tests have failed to reliably detect these early neuromuscular deficits in the hind-limbs of the SOD1$^{G93A}$ mice. Recently, a novel gait analysis technique performed on a similar mouse model of ALS revealed behavioural deficits in pre-symptomatic mice (Wooley et al., 2005). In the SOD1$^{G93A}$ mouse an early deficit was also uncovered when the mice were made to exercise; 56-day old SOD1$^{G93A}$ mice were only able to run at a speed of 12m/min, but mice with a delayed disease onset could run at speeds of 16 m/min (Veldink et al., 2003). We have shown that by 56-days of age, half the motor units innervating the TA muscle have already been lost, resulting in significant deficits in the force produced by the TA muscle (Fig 3-6). Given that the TA muscle is proportionally activated with increases in treadmill speeds (Roy et al.,

1991), TA muscle deficits should become increasingly apparent with faster running speeds. Novel functional tests that measure changes in stance and locomotion need to be implemented to fully assess neuromuscular function in the SOD1$^{G93A}$ mouse.

In conclusion, we show that clinically detectable muscle deficits are present at the time of earliest pathological dysfunction in the SOD $^{G93A}$ mouse model of fALS. Our findings provide convincing evidence that motor unit loss and associated muscle function precedes motoneuron death in accordance with the "die-back" hypothesis (Fischer et al., 2004). Moreover, our time-course of preferential motor unit loss reconciles previous contradictory reports of pre-symptomatic motor unit loss in the SOD1 mouse model of ALS. Anatomical evidence has suggested that certain motoneurons and axons are more resilient in the SOD1$^{G93A}$ mice (Frey et al., 2000;Schaefer et al., 2005), but ours is the first report providing electrophysiological evidence of preferential motor unit loss. Further study is needed to examine the mechanisms underlying this preferential motor unit loss. Here, we have shown that the largest motoneurons with the biggest axon calibers are the most vulnerable to cell death, implicating size as contributing factor for selective vulnerability. These big motoneurons also have the largest innervation ratios (Gordon et al., 2004), and the smallest complement of oxidative enzymes (Ishihara et al., 1995), suggesting that activity and antioxidant capability may also play a role in the loss of functional motor units. Surprisingly, we did not find any evidence of compensatory motor unit expansion in any of the hindlimb muscles studied, as evidenced by the parallel decline in

motor unit numbers and whole muscle contractile force. Despite mice appearing asymptomatic, deficits in whole muscle contractile force were detected in the fast-twitch muscles as early as 40-days of age, highlighting the need of more sensitive behavioural tests that can detect subtle muscle deficits in the hindlimbs.

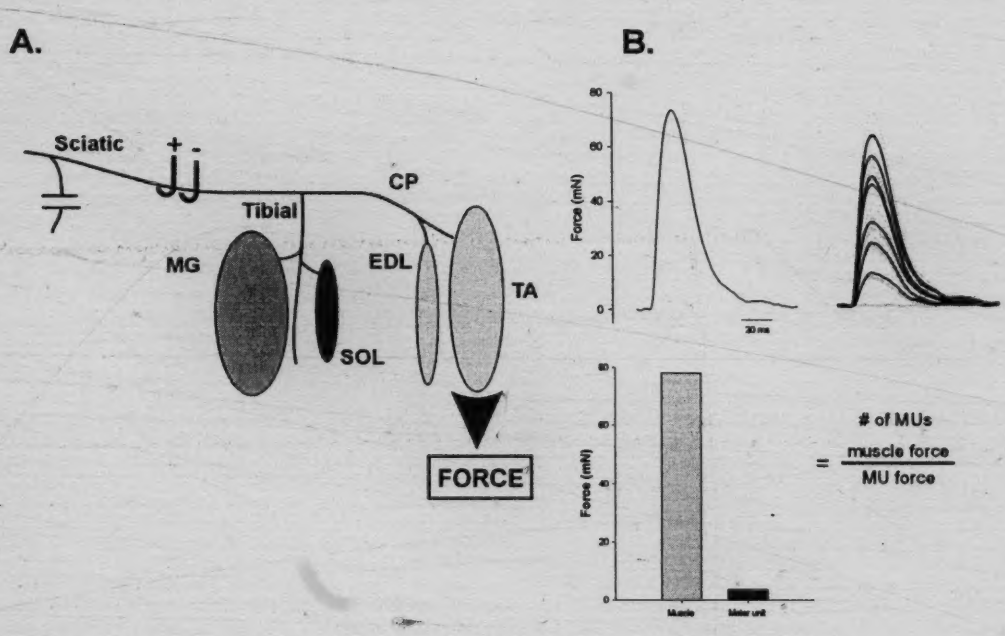


***Figure 3-1*** Schematic illustrating the procedure of recording of whole muscle and motor unit isometric contractile forces from the hind-limb muscles of mice. *(A)* Tendons of the MG, SOL, EDL and TA muscles were exposed and individually attached to the force transducer in order to record the contractile force generated by stimulating the sciatic nerve through silver wire electrodes. *(B)* The sciatic nerve was supramaximally stimulated to elicit maximal isometric twitch- and tetanic- contractile forces in each of the hind-limb muscles. Twitch force from a $SOD1^{WT}$ TA muscle is shown. The stimulus voltage was then reduced so that no force was elicited. The voltage was then slowly increased, resulting in the incremental increases in the whole muscle isometric force. Each increment in whole muscle force was assumed to be the force associated with the addition of one motor unit. A computer randomly selected 8 to 15 increments, which were averaged to yield the mean motor unit force. Motor unit number was calculated by dividing the mean motor unit force into the whole muscle twitch.

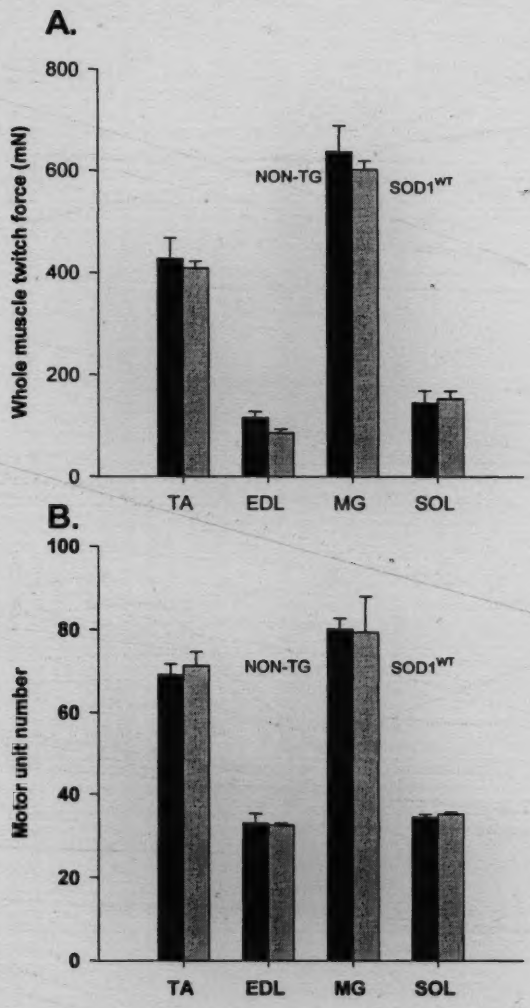


***Figure 3-2*** Comparison of the whole muscle twitch force (*A*) and calculated motor unit numbers (*B*) in the hind-limb muscles of 120- day old non-transgenic wild-type (black bars) and $SOD1^{WT}$ (grey bars). There were no significant differences in either whole muscle twitch force or motor unit number between the non-transgenic and $SOD1^{WT}$ in any of the four muscles we studied. Data is reported as means $\pm$ SEM. * Significantly different ($p< 0.05$), ** significantly different, $p<0.01$.

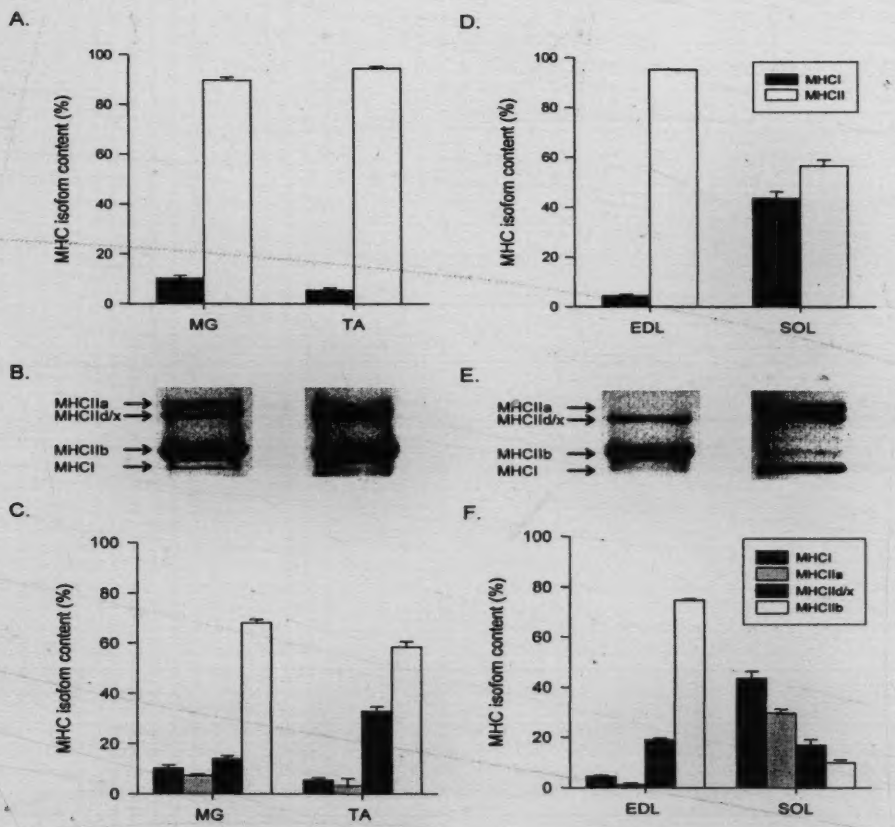


***Figure 3-3*** The proportion of muscle fibers expressing slow and fast MHC isoforms in the hindlimb muscles of 80-day old $SOD1^{WT}$ mice. *(A)* In the MG, TA and EDL muscles, muscle fibers expressed predominantly fast MHC II isoforms. In contrast, the SOL muscle had much higher proportions of slow MHC I isoforms. *(B)* Representative SDS-PAGE gels showing the MHC isoforms. *(C)* Proportions of the different subtypes of MHC isoforms in the MG and TA muscles and in the *(D-E)* EDL and SOL muscles of the $SOD1^{WT}$ mice. Data is presented as mean ± SE.

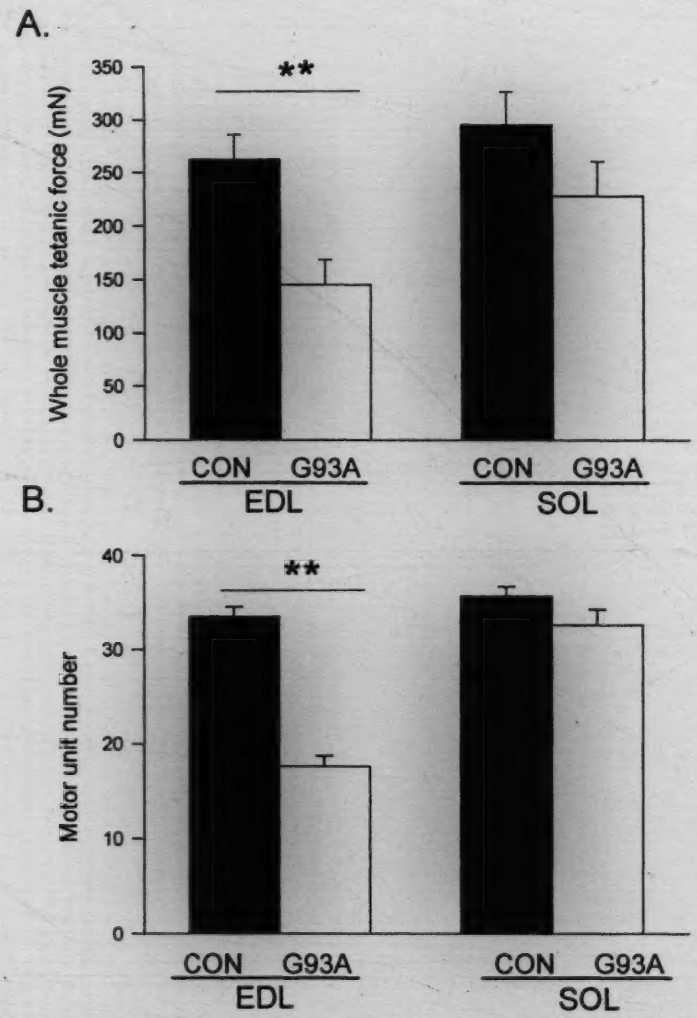


***Figure 3-4*** Whole muscle isometric tetanic forces and the number of intact motor units in the $SOD1^{G93A}$ mouse EDL and SOL muscles at 80-days of age. *(A)* The whole muscle tetanic contractile force developed by the 2 muscles indicates that there was a significant decline in the twitch force generated by the EDL, but not the SOL, in the $SOD1^{G93A}$ mouse as compared to control mouse muscles. The tetanic force produced by the $SOD1^{G93A}$ mouse was approximately half of the control EDL muscle values. *(B)* The number of motor units in the EDL muscle also declined to half of the number in the control EDL muscle.

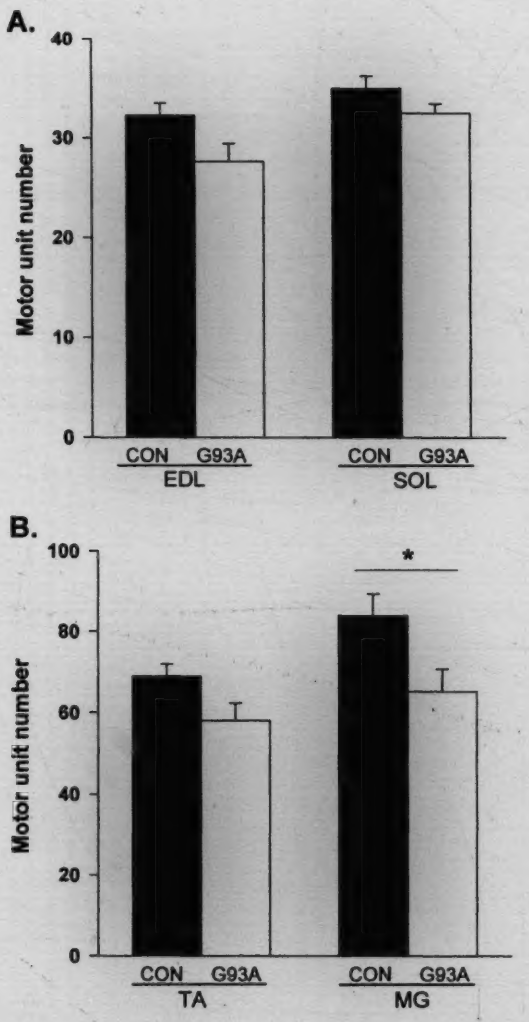


***Figure 3-5*** The number of intact motor units in pre-symptomatic 40-day old SOD1$^{G93A}$ and age-matched control (CON) mice. *(A)* As compared to controls, the number of motor units in the EDL, but not SOL muscle, is lower in the SOD1$^{G93A}$ mouse at 40-days of age. *(B)* The number of intact motor units iwas significantly decreased in the SOD1$^{G93A}$ mouse MG, but not TA, muscles, as compared to controls.

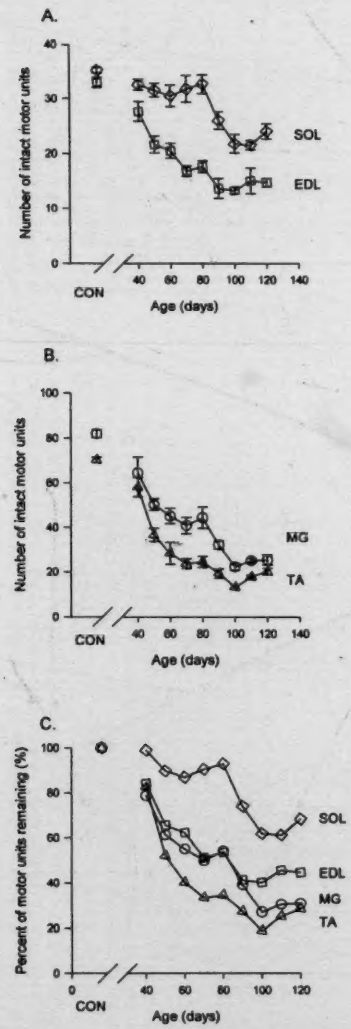


***Figure 3-6*** The time course of motor unit loss in the SOD1$^{G93A}$ mice, from 40- to 120-days of age. The number of motor units in the control mice did not change throughout the life-span of the SOD1$^{G93A}$ mouse, and control values are presented as averages of 3 time-points, 40-, 80- and 120-days of age. (*A)* There was no loss of motor units from the SOL muscle until 90-days of age, coincident with the reported onset of symptoms. In the EDL, there was an exponential loss, with an initial steep decline of motor units. (*B)* Motor units are also exponentially lost from the TA and MG muscles, with a slight biphasic effect. Motor units are lost quickly from 40 to 60 days of age, and again from 80 to 100 days of age. (*C)* The percent of motor units left, as compared to control muscles. In all 4 hind-limb muscles the initial decline in motor unit numbers is very steep, but the number of motor units reaches a plateau in the symptomatic stage of disease, with no motor units being lost after 100-days of age (*n* was ~4-5 at each time point, see table 3-1 for details).

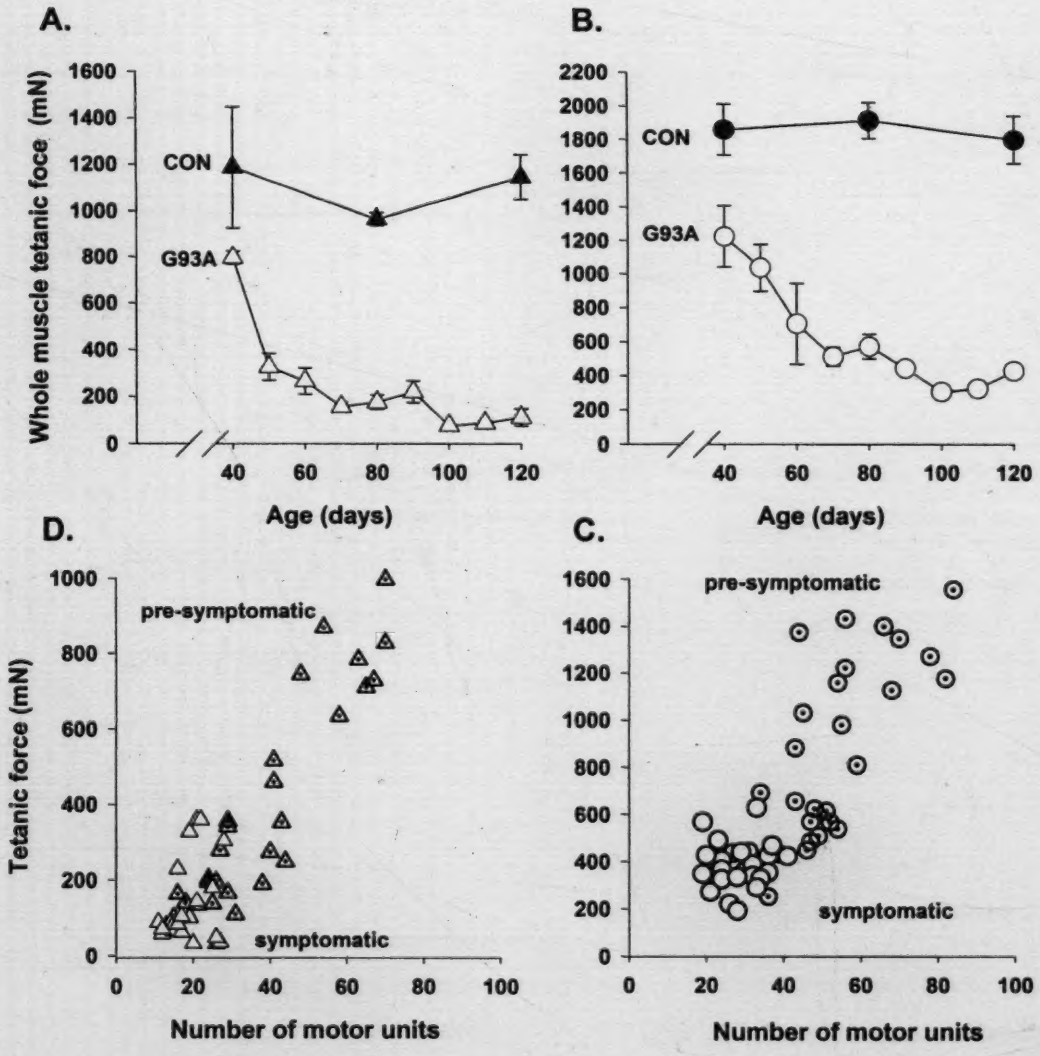


***Figure 3-7*** The whole muscle isometric tetanic forces produced by the MG and TA muscles in SOD1$^{G93A}$ as compared to control mice. Tetanic force was measured at 10-day intervals from 40- to 120-days of age in the SOD1$^{G93A}$ mice. *(A)* The tetanic contractile force generated by the control TA muscle stayed constant throughout he life-span of the SOD1$^{G93A}$ mouse. In the SOD1$^{G93A}$ mice, the tetanic force declined exponentially in the TA muscle. *(B)* The same exponential decline was seen in the tetanic force produced by the SOD1$^{G93A}$ mouse MG muscle, as compared to the unchanging tetanic force produced by the control mouse MG. Data in *A* and *B* are reported as mean ± SEM. (*n*= 4-12 at each time point, see methods for details). *C* and *D* In the TA and MG muscles of the SOD1$^{G93A}$ mouse the tetanic force generating capacity and the number of motor units declines in parallel (symbols with dots are pre-symptomatic mouse values, open symbols are symptomatic mouse values). *(C)* The tetanic force is plotted against the number of motor units remaining in the TA muscle.

***Table 3-1*** Estimates of the number of functional motor units in 3-11 SOD1-G93A mice were made for each of the 4 muscles at 10 day intervals.

| | Age (Days) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Muscle | 40 | 50 | 60 | 70 | 80 | 90 | 100 | 110 | 120 |
| SOL | 4 | 4 | 4 | 5 | 5 | 10 | 4 | 4 | 6 |
| EDL | 3 | 5 | 4 | 5 | 5 | 10 | 4 | 4 | 6 |
| MG | 5 | 5 | 4 | 5 | 5 | 11 | 4 | 4 | 6 |
| TA | 4 | 6 | 4 | 5 | 4 | 8 | 4 | 4 | 6 |

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# Chapter 4

# THE EFFECTS OF GENDER ON PROGRESSIVE MOTOR UNIT LOSS IN A TRANSGENIC MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS (ALS)

## 4.1 INTRODUCTION

In amyotrophic lateral sclerosis (ALS) there is a progressive loss of functional motor units, resulting in weakness and eventual paralysis (Chapters 2-3; Gurney et al., 1994). There is a male predominance of the disease until the age of 60 years of age, with a male to female ratio of 1.8 to ~1 until menopause (Cashman et al., 2000;Strong and Rosenfeld, 2003), and an older average age of onset reported for females (Rudnicki, 1999). The development of transgenic mouse models has allowed us to explore how gender affects disease progression; in the $SOD1^{G93A}$ transgenic mouse model of ALS, mice develop weakness and tremors by 90 days of age, and are completely paralysed by ~120 days of age (Chiu et al., 1995), with female mice becoming symptomatic later than males (Trieu and Uckun, 1999;Veldink et al., 2003).

We have previously reported that there is a muscle-specific loss of the largest motor units as early as 40 days of age in male $SOD1^{G93A}$ mice (Chapter 3) indicative of an axonal die-back pathology (Fischer et al., 2004;Schaefer et al., 2005). Here, we explore whether there are gender-mediated differences in the progressive and selective motor unit loss in $SOD1^{G93A}$ mice to account for the delayed symptom onset.

## 4.2 METHODS

### 4.2.1 Generation of transgenic mice

Transgenic male mice expressing a high copy number of the mutant Cu/Zn superoxide dismutase (SOD1) gene with a glycine to alanine base pair mutation at the 93$^{rd}$ codon (SOD1$^{G93A}$; B6JSL-TgN(SOD1-G93A) were obtained from Jackson Laboratories, and were bred and identified as described elsewhere (Chapters 2-3; (Rosen et al., 1993;Gurney et al., 1994). The University of Alberta Health Sciences Animal Policy and Welfare Committee approved all protocols. Electrophysiological recordings were carried out at 10 day intervals on both male and female SOD1$^{G93A}$ mice. Approximately 4 to 5 of each gender were studied at each time point (the exact numbers are reported in table 4-1).

### 4.2.2 Electrophysiological recordings

Motor unit number and contractile forces were measured as previously described (Chapter 3). In brief, under anaesthesia the tibialis anterior (TA), extensor digitorum longus (EDL), medial gastrocnemius (MG) and soleus (SOL) muscle tendons were isolated for individual attachment to a strain gauge. The sciatic nerve was stimulated through silver wire electrodes, and the resultant muscle contractile forces were recorded by attachment to a Kulite strain gauge, amplified, and digitized using axoscope (Version 8.0, Axon Instruments, USA). To estimate the number of motor units, controlled graded electrical stimulus (100 μs duration) was applied to the sciatic nerve at a frequency of 0.5 Hz, and the

resulting all-or-none incremental increases in isometric force were recorded. These incremental increases in force represent isometric twitch force associated with the addition of one motor unit (McComas, 1995). Using MATLAB (Mathworks), all the digitized isometric twitch force recordings were over-laid and the individual motor unit forces that were associated with 10-15 randomly chosen motor units were calculated using template subtraction. The ratio of the maximal twitch force to the average motor unit twitch force was calculated to give an estimate of the number of motor units, as previously described by McComas (1971).

### 4.2.3 Statistical analysis

Data are presented as means $\pm$ standard errors (SE). Statistical significance between experimental groups was assessed Students' t-test in Sigma Plot (SPSS, Version 8.0), with differences being considered statistical significant if $p<0.05$. In the Figures significant is denoted by asterixes, with * for $p<0.0.5$ and **for $P<0.03$. Power calculations were made to confirm sufficient power and/or sample size using PC-Size (STATOOLS, Version 2.13, 1986). Power values of at least 0.8 were required to consider results significant.

## 4.3 RESULTS

### 4.3.1 Motor unit number

In both male and female SOD1$^{G93A}$ mice, the number of motor units in the fast-twitch TA, EDL and MG hindllimb muscles fell steeply in the pre-symptomatic phase of disease (Fig 4-1*A-C*). In contrast, there was no significant decline in the number of motor units in the slow-twitch ankle extensor, the SOL, until 90 days of age (Fig 4-1*D*). This early and selective decline in the number of intact motor units was unaffected by the gender of the mice; the magnitude and rate of the motor unit loss was not significantly different in the EDL and SOL muscles of presymptomatic male and female SOD1$^{G93A}$ mice at any time point. In symptomatic animals, there were significantly more motor units in the male EDL and SOL muscles as compared to female muscles at 120 days of age ($p<0.05$, Fig 4-1*C*,D).

When motor units were enumerated in the EDL, TA and MG muscles throughout the entire lifespan of SOD1$^{G93A}$ mice, we found that there was a trend for the initial motor unit loss to be more gradual in pre-symptomatic female SOD1$^{G93A}$ muscles, as compared to males. That is, in the TA and EDL muscles, there appeared to be more intact motor units in the female than the male muscles from 50 to 70 days of age (Fig 4-1*A,C)*. Female mice also had significantly more intact MG muscle motor units at 50 days of age, but there were no other significant gender-dependent differences in the number of intact MG motor units (Fig 4-1*B*).

### 4.3.2 Contractile muscle force

There was a parallel decline of whole muscle isometric twitch force and motor unit number in the TA and MG muscles in both male and female SOD1$^{G93A}$ mice (Figs 4-1 and 4-2). Had there been a compensatory increase in the force produced by the remaining intact motor units, the tetanic force would have been maintained until 75 - 85% of the motor units were lost; in conditions of partial denervation motor units in rats and cats can enlarge approximately by a magnitude of 5 (Gordon et al., 1993;Rafuse et al., 1992). The proportionate decline in muscle tetanic force and motor unit numbers in the fast-twitch hindlimb muscles of the SOD1$^{G93A}$ mice indicated a lack of functional compensation for motor unit loss, regardless of gender.

At 40 days of age, the contractile force produced by male TA muscles was significantly greater than the forcefulness of female TA muscles in SOD1$^{G93A}$ mice (Fig 4-2*A*). There were no other gender-dependent significant differences in the forcefulness of TA and EDL muscles throughout the lifespan of SOD1$^{G93A}$ mice until 120 days of age. At this end stage of disease the TA muscle of male SOD1$^{G93A}$ was significantly more forceful than female TA muscles. In the EDL muscle there were no significant gender-dependent differences in contractile force throughout the lifespan of the SOD1$^{G93A}$ mouse. In pre-symptomatic SOD1$^{G93A}$ mouse MG muscles there were no significant gender-dependent differences in whole muscle force (Fig 4-2*B*). As in the TA muscle, male

SOD1$^{G93A}$ mouse MG muscles were more forceful than females at 120 days of age.

Throughout much of the disease progression, the forcefulness of female SOD1$^{G93A}$ mouse SOL muscles tended to be lower than in male SOD1$^{G93A}$ mice. The difference in the forcefulness of SOL muscles in SOD1$^{G93A}$ male and female mice was significant at 50, 70 and 80 days of age (Fig 4-2*D*). Once mice became symptomatic, and there was a loss of motor units from SOL muscles, there were no gender dependent differences in the forcefulness of this slow-twitch muscle. Importantly, the forcefulness of the SOL muscle did not decline in proportion to the loss of motor units, indicating that there might be some compensatory processes maintaining the strength of postural, slow twitch muscles in symptomatic SOD1$^{G93A}$ mice.

## 4.4 DISCUSSION

Here we show that gender minimally affects the progressive motor unit loss and associated decline in the forcefulness of hindlimb muscles in SOD1$^{G93A}$ mice at certain time points throughout disease progression (Fig 4-1). In the fast-twitch TA and MG muscles the number of motor units in both genders declined proportionately with whole muscle isometric force, indicating that there was no functional compensation for the motor unit loss (Fig 4-2).

Gender has been reported to influence time of disease onset in SOD1$^{G93A}$ mice (Trieu and Uckun, 1999;Veldink et al., 2003;Miana-Mena et al., 2005). The female sex hormone estrogen is a potent antioxidant that can act via non-estrogen receptor mediated pathways to reduce selective motoneuron death triggered by excess glutamate *in vitro* (Nakamizo et al., 2000); pre-treatment with estrogen also protects motoneurons cultured from SOD1$^{G93A}$ mice, which are especially sensitive to glutamate excitotoxicity (Kruman et al., 1999). *In vivo,* disease progression in SOD1$^{G93A}$ mice can be accelerated in female mice by ovarectomy, which in turn can be reversed by the administration of estrogen (Groeneveld et al., 2004). In addition to its neuroprotective effects, estrogen can also protect muscles from the oxidative stress (Feng et al., 2004;Persky et al., 2000).

The protection conferred by estrogen to motoneurons and muscles may be responsible for the trend indicating an increased number of motor units in female TA, MG and EDL muscles, as compared to male SOD1$^{G93A}$ mouse muscles in pre-symptomatic animals (Fig 4-1). However, our results indicate that

gender and the associated differences in estrogen concentrations fail to have an impact on the overall progressive loss of motor units in any of the muscles; the rate and extent of motor unit loss over the entire disease course do not appear to vary according to gender (Fig 4-1). Veldink et al (2003) also reported that muscle morphology and motoneuron numbers are not significantly different in male and female SOD1$^{G93A}$ mice, despite significant earlier onset in male mice.

In human ALS patients, gender fails to affect survival time (Magnus et al., 2002) and estrogen replacement therapy does not confer a survival benefit to menopausal women with ALS (Rudnicki, 1999). Other antioxidant and antiglutaminergic drugs, besides estrogen, also have had only limited success in the treatment of ALS; the antiglutaminergic drug Riluzole prolongs survival time by a mere two months (Miller et al., 2003). This failure to halt or prevent ALS disease progression by reducing oxidative stress and glutamate excitotoxicity suggests that the accumulation of reactive oxygen species and glutamate may not be the primary events provoking selective motoneuron demise in ALS. Indeed, in the SOD1$^{G93A}$ mouse model of fALS, oxidative stress in the spinal cord is evident only after disease onset coincident with the loss of motoneurons from the lumbar spinal cord (Chiu et al., 1995). As functional motor units are lost at least 50-days prior to motoneuron death in a die-back fashion (Chapter 3), it is likely that oxidative stress does not cause this early motor unit dysfunction. Recently, it has been hypothesized that protein aggregation, disruption of axonal transport and inflammation, along with a host of other factors may interact to result in motoneuron death in ALS (Cleveland and Rothstein, 2001;Shaw, 2005).

Gender also does not appear to influence the functional adaptability of the lower motoneurons as there appears to be little compensation for motor unit loss in both male and female $SOD1^{G93A}$ mice. This lack of compensation is indicated by the parallel decline in whole muscle force and motor unit numbers in the fast-twitch muscles (Chapter 3). The reason why disease may be delayed in female $SOD1^{G93A}$ mice (Veldink et al., 2003), even though gender only minimally affects motor unti loss and the ability of motor units to compensate, may be related to an increase in the plasticity of other systems. For example, functional compensation can also be mediated by the recruitment of upper motoneurons. It now remains to be seen whether changes in the output of upper motoneurons is affected by gender in the $SOD1^{G93A}$ mice.

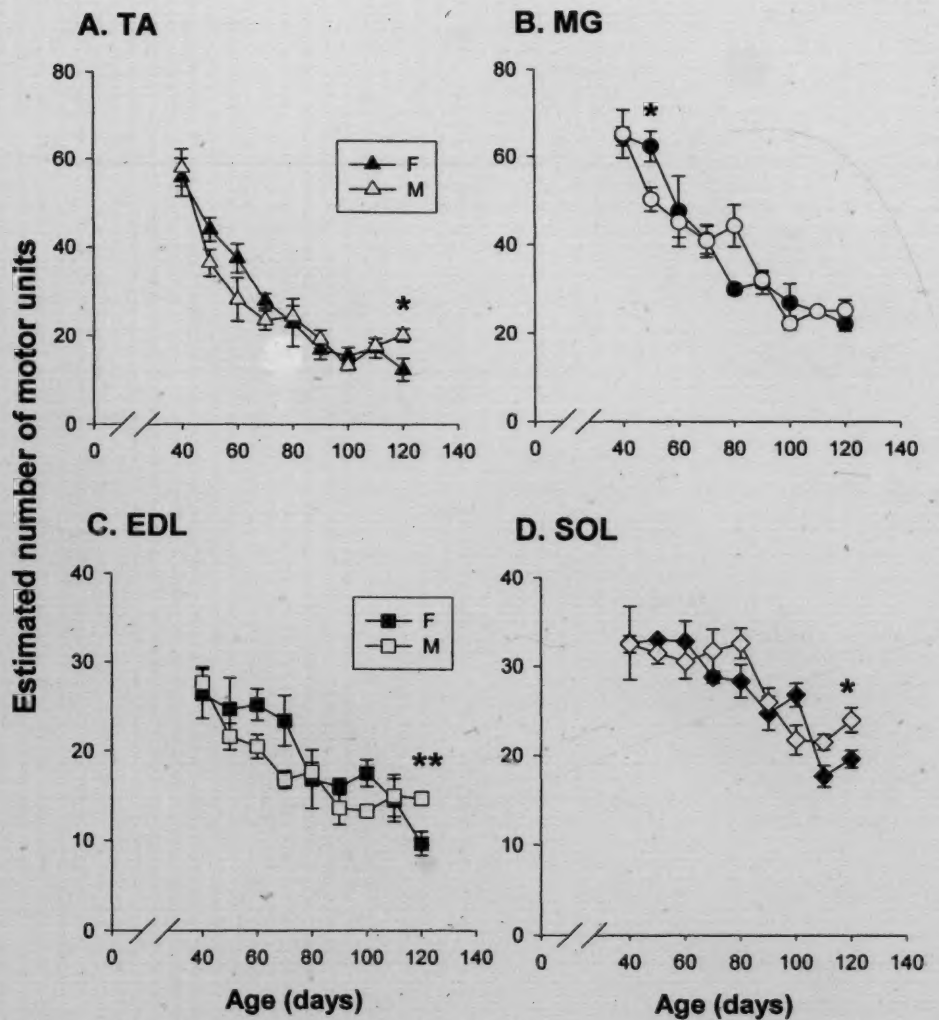


**Figure 4-1** The number of intact motor units in the TA (*A*), MG (*B*), EDL (*C*) and SOL (*D*) muscles of male and female $SOD1^{G93A}$ mice. Closed symbols are for female mice, and open for male mice. The data is presented as mean ± standard error (SE), with significance denoted by asterixes. (*) indicates $p<0.05$ and (**) indicates $p<0.01$. We recorded from 3-11 mice at each time point, see table 4-1 for exact numbers.

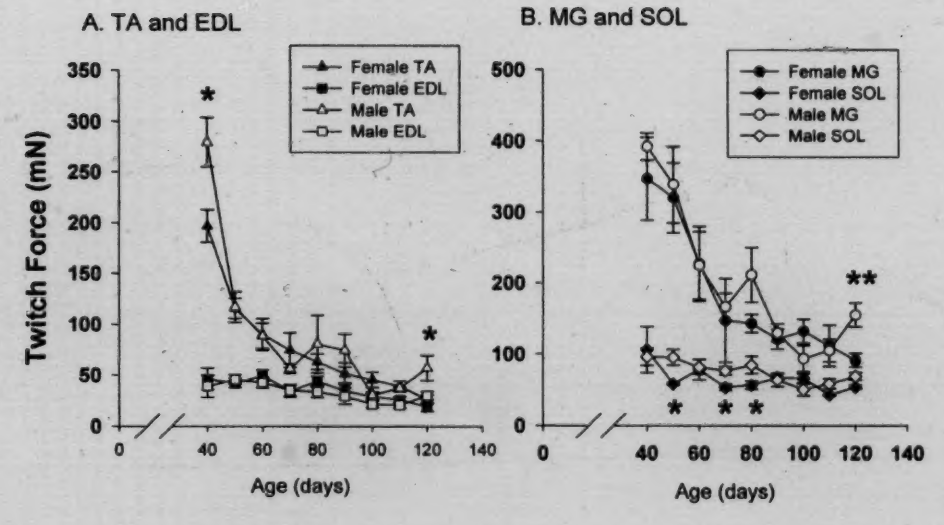


**Figure 4-2** Whole muscle twitch force in the ankle extensors *(A)* and flexors *(B)* of male and female SOD1$^{G93A}$ mice.

**Table 4-1** Estimates of the number of motor units in each of 4 muscles were made at 10 day intervals for ~4 to 5 SOD1G93A male (M) and female (F) mice.

| | Age (Days) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 40 | | 50 | | 60 | | 70 | | 80 | | 90 | | 100 | | 110 | | 120 | |
| Muscle | M | F | M | F | M | F | M | F | M | F | M | F | M | F | M | F | M | F |
| SOL | 4 | 3 | 4 | 2 | 4 | 6 | 5 | 5 | 5 | 6 | 10 | 4 | 4 | 5 | 4 | 3 | 6 | 5 |
| EDL | 3 | 5 | 5 | 3 | 4 | 5 | 5 | 5 | 5 | 7 | 10 | 7 | 4 | 5 | 4 | 4 | 6 | 5 |
| MG | 5 | 5 | 5 | 3 | 4 | 6 | 5 | 5 | 5 | 7 | 11 | 7 | 4 | 5 | 4 | 4 | 6 | 5 |
| TA | 4 | 5 | 6 | 3 | 4 | 6 | 5 | 6 | 4 | 6 | 8 | 7 | 4 | 5 | 4 | 4 | 6 | 5 |

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# Chapter 5

# FUNCTIONAL OVER-LOAD SAVES MOTOR UNITS IN THE SOD1-G93A TRANSGENIC MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS (ALS)

## 5.1 INTRODUCTION

The selective and progressive loss of motoneurons in amyotrophic lateral sclerosis (ALS) results in skeletal muscle denervation, which manifests as weakness and eventually paralysis. Recent studies on exercise in ALS patients have shown a reduction in spasticity (Ashworth et al., 2006) and a mild improvement in motor deficits and general quality of life (Drory et al., 2001); (Krivickas, 2003). However, the benefit of increased activity and exercise in patients with ALS is still contentious, in part because some epidemiological studies have suggested that involvement in organized sports may increase the likelihood of developing ALS (Scarmeas et al, 2002; Chio et al, 2005). It is important to resolve the effects of increased neuromuscular activity on motor units in ALS before a program of exercise can be prescribed to ALS patients.

The transgenic mouse model of ALS was developed by expressing a mutant gene associated with ~20% of all familial ALS cases in transgenic mice. The gene encodes cytosolic Cu/Zn superoxide dismutase (SOD1), which is an antioxidant responsible for the conversion of superoxide anion to hydrogen peroxide (Rosen et al., 1993). The most common mutant form of SOD1 has a glycine to alanine conversion at the 93$^{rd}$ codon (SOD1$^{G93A}$) which is thought to

confer a yet to be determined gain of cytotoxic function to SOD1 (Strong and Rosenfeld, 2003). Mice expressing high copy numbers of the human $SOD1^{G93A}$ gene ($SOD1^{G93A}$ mice) develop symptoms and pathology similar to human patients (Gurney, 1994). Motoneurons are known to be lost in a "die-back" manner in the $SOD1^{G93A}$ mouse model of ALS; denervation of muscle fibers, loss of axons and thinning of nerves in the ventral roots all precede the loss of motoneurons from the lumbar spinal cord (Fischer et al., 2004;Chiu et al., 1995;Frey et al., 2000). Motoneuron loss from the lumbar spinal cord has been successfully retarded, and lifespan extended by allowing mice to voluntarily run on treadmills during the disease course (Kaspar et al., 2005). In contrast when mice were made to exercise in short bursts with high intensity, motor deficits and disease onset were hastened (Mahoney et al., 2004). There are at present no studies that examine the effects of exercise on muscle force production or on the viability of functional motor units.

We have previously shown that die-back preferentially affects the larger motoneurons that innervate the largest, most forceful type IIB muscle fibers (Chapter 2; Henneman, 1985). The cellular mechanisms underlying this preferential and biphasic decline of functional motor units in ALS has not yet been fully elucidated. We have previously hypothesized that the initial, dramatic loss of motor units from fast-twitch hindlimb muscles in the $SOD1^{G93A}$ mice necessitates the increased recruitment of remaining motor units to compensate for motoneuron die-back in pre-symptomatic mice (Chapter 2). This compensatory increase in neuromuscular activity may result in the conversion of

muscle fibers from IID/X to IIA phenotypes. Evidence of activity dependent conversion is apparent already at 60 days of age in the TA muscle of male $SOD1^{G93A}$ mice (Chapter 2). As the motoneurons which innervate type I and IIA muscle fibers are less vulnerable to die-back, increased recruitment and the associated conversion of muscle fiber phenotypes confers protection upon the remaining motoneurons (Chapter 3). In support of this theory, experimentally increasing motor unit activity by allowing mice free access to rodent exercise wheels prolongs the lifespan of $SOD1^{G93A}$ mice by ~25 days and also attenuates the loss of motoneurons from the lumbar spinal cord (Kaspar et al., 2005). Functional motor units have not yet been characterized or enumerated in $SOD1^{G93A}$ mice following any exercise regime. Here we investigate whether wheel running for 40 days prior to the onset of severe muscle weakness and paralysis has any effect on the number of functional motor units in $SOD1^{G93A}$ mice.

## 5.2 METHODS

### 5.2.1 Generation of SOD1$^{G93A}$ mice

Male transgenic mice that express mutant human SOD1$^{G93A}$ genes (B6JSL-TgN (SOD1-G93A)) were purchased from Jackson Laboratories, USA. A colony was established by breeding SOD1$^{G93A}$ male mice to non-transgenic B6JSL female mice. We identified transgenic SOD1$^{G93A}$ mice from the offspring of these matings through standard PCR protocol for the human SOD1 performed on ear samples taken at the time of weaning (Rosen et al., 1993). After the pups were weaned at ~21 days of age they were separated by gender into standard rodent cages with free access to food and water. All of the experiments were approved by the University of Alberta Health Sciences Laboratory Animal Ethics committee and were carried out in accordance with the guidelines of the Canadian Council for Animal Care.

### 5.2.2 Voluntary Wheel Running

A total of 22 SOD1$^{G93A}$ mice (8 males and 14 females) were given free access to running wheels from 40 to 80 days of age. Standard small rodent exercise wheels were placed in rat cages covered with raised filter tops instead of standard wire tops to allow free movement of exercise wheels. Rodent chow and water were provided in small dishes placed on the floor of the cage. The mice were separated according to gender and housed two to a cage, with one wheel being allocated to each animal. For control animals, we randomly selected 13 SOD1$^{G93A}$ mice (5 males and 8 females) to be housed in normal mouse cages between 40 to 80 days age. For both the experimental and the control groups,

motor unit recordings were carried out at 80 days of age before the appearance of overt weakness in the $SOD1^{G93A}$ mouse hindlimbs.

### 5.2.3 Partial Denervation of hindlimb muscles

We partially denervated the hindlimbs of 32 mice (22 $SOD1^{G93A}$ mice and 10 age matched wildtype control mice) by avulsing one of the main spinal roots that innervates the hindlimbs at 40 days of age. Mice were anaesthetized by an intraperitoneal (IP) injection of a cocktail made up of ketamine (100mg/mL) and atravet (10mg/mL) diluted in sterile saline. The dosage of the anesthetic was 17.5mL/kg body weight for both control and $SOD1^{G93A}$ mice. A small incision was made along the spinal cord just above the iliac crest to expose the nerves that radiate from the ventral roots. From the iliac crest, we counted the ventral roots to identify the nerves coming from the L4 and the L5 segments on the right side of the mouse. Using fine forceps and scissors, the bone around the segment of interest was cleared of muscle, and the spinal root was firmly grasped and avulsed to prevent regeneration. Mice were sutured and allowed to recover before being returned to their home cages for the next 50 days. All experimental animals were subject to daily health checks during which the general health and mobility of all experimental animals were monitored. Our electrophysiological recordings revealed that partial denervation of the L4 ventral root did not cause a significant decrease in the number of motor units in the EDL muscle in control wildtype animals. We therefore did not involve the EDL muscle in our analysis for this series of experiments on the effects of partial denervation.

### 5.2.4 Electrophysiological Recordings

*Surgery* The method used to enumerate and characterize motor units was described in Chapter 3. In brief, mice were anesthetized with the ketamine/atravet cocktail injected IP to induce surgical aneasthesia. Periodically, additional doses of the cocktail were administered IP in order to maintain aneasthesia throughout the experiment. The lower hindlimbs of the animals were exposed, and the tendons to the plantaris and lateral gastrocnemius muscles were identified and cut. Tendons to the soleus (SOL), TA and extensor digitorum longus (EDL) muscle were next identified and separated so that they could be tied individually with a 4.0 silk thread for attachment to the strain gage (Kulite model KH-102) during recording. Following isolation of the nerves bilaterally, the sciatic nerves were exposed on both sides, and wire electrodes were sutured alongside the nerve for stimulation. Both hindlimbs were prepared in all animals, but in the exercise paradigm we would normally record only from one limb, unless there was a problem with the immobilization of the leg. In animals where one of the hindlimbs had been partially denervated we recorded bilaterally, and used the non-operated side as a control. To immobilize the legs, we clamped the hindlimbs at the knees and ankles, being careful not to interfere with the blood supply to the muscles.

*Isometric force recordings and motor unit enumeration.* Isometric forces evoked from individual muscles through electrical stimulation of the sciatic nerve were amplified and digitized using Axoscope Software (version 8.0, Axon Instruments,

USA). The lengths of the muscles were adjusted to yield maximal evoked isometric twitch tension in response to stimulation of the sciatic nerve. Maximal contractile muscle forces were recorded in response to single suprathreshold (2X threshold amplitude) stimulation of the sciatic nerve at a frequency of 0.5 Hz.

Average motor unit force was determined by incremental stimulation of the sciatic nerve to elicit discrete increases in motor unit force. Each of these discrete increments in force is attributed to the recruitment of a motor unit. The incremental increases in whole muscle force were recorded in response to sciatic nerve stimulation at a frequency of 0.5 Hz, and the amplitude of which was manually controlled from 0 to 10 V. The all-or-none increments in muscle force were recorded and overlaid in a custom software program written in MatLab. Using this program 8-20 increments were randomly chosen from throughout the entire range of muscle forces. We calculated average motor unit force as the mean force associated with these increments. In order to estimate the number of intact motor units, the whole muscle twitch contractile force was divided by the average motor unit force.

### 5.2.5 Statistical analysis

Data was considered significant if $p \leq 0.05$, and p was determined using t-test (SPSS version 14.0, 2005). In the figures significance is denoted by stars (* for $p \leq 0.05$ and ** for < 0.01). For calculations including motor unit forces we tested the data set for normalcy using a one-sided Kolmogrov-Smirnov test. For non-normally distributed data significance was then determined using the Mann-Whitney U Test in the SPSS program.

## 5.3 RESULTS

### 5.3.1 Preferential motor unit loss at in SOD1$^{G93A}$ mice around the time of symptom onset

In the current paper we determined whether increased neuromuscular activity affects the progressive loss of motor units in a transgenic mouse model of ALS. The first set of experiments involved voluntary wheel running from 40 to 80 days of age for SOD1$^{G93A}$ mice. In normally caged SOD1$^{G93A}$ mice, there is significant motor unit loss from the hindlimb muscles during this time period (40 to 80 days; Chapter 3) despite mice remaining "asymptomatic" until ~90 days of age (Veldink et al., 2003;Chiu et al., 1995). By 80 days of age a large proportion of the motor units innervating the fast-twitch hindlimb muscles was lost (n=5 for males, n=8 for females; Fig 5-1). In the TA muscle of 80 day old SOD1$^{G93A}$ mice, only ~30% of the functional motor units remained, as compared to wildtype control mouse TA muscles (Fig 5-1*A*). In the MG and EDL muscles, approximately half the functional motor units were lost (Fig 5-1*B, C*). In contrast, there was very little loss from slow-twitch hindlimb muscles at 80 days of age; in the SOL muscle of SOD1$^{G93A}$ mice more than 95% of the motor units remained intact. (Fig 5-1*D*).

In SOD1$^{G93A}$ mice, gender reportedly influences the onset of disease symptoms (Kirkinezos et al., 2003;Veldink et al., 2003), but not the progressive loss of motor units throughout the entire disease course (Chapter 4). At 80 days of age there were no gender dependent differences in the number of motor units innervating the TA, EDL and SOL muscles in SOD1$^{G93A}$ mice. Male SOD1$^{G93A}$

mice did have significantly more intact motor units than females at 80 days of age in the MG muscle (Fig 5-1*B*). At 90 days of age, when all SOD1$^{G93A}$ are symptomatic we found no gender-related differences in the proportion of remaining motor unit in any of the hindlimb muscles (n=12 for males, n=9 for females; Fig 5-1).

Despite the overt behavioral changes that occur between 80 and 90 days of age, there was no further motor unit loss during this 10 day period from the TA and EDL muscles of either male or female SOD1$^{G93A}$ mice (Fig 5-1*AC*). In the MG and SOL muscles, there were significant motor unit losses between 80 and 90 days in male SOD1$^{G93A}$ mice (Fig 5-1*BD)*. Motor unit loss significantly slows in the latter, symptomatic stage of disease in the SOD1$^{G93A}$ mice as compared to earlier, pre-symptomatic stages of disease (Chapter 2). The biphasic loss of functional motor units in SOD1$^{G93A}$ mice parallels the disease process in human ALS patients, where motor unit loss is fastest in the first 6 months after a motoneuron pool is first affected (Dantes and McComas, 1991).

### 5.3.2 Voluntary wheel running has gender dependent effects

To determine how increased neuromuscular activity affects the survival and forcefulness of motor units in the SOD1$^{G93A}$ mouse model of ALS we allowed the mice free access to standard rodent wheels. When non-transgenic normal mice were given access to exercise wheels male mice ran for shorter durations and covered less distance than their female counterparts (De Bono et al., 2006). Despite the differences in spontaneous activity, only male mice lost a significant

amount of body weight and ankle flexor muscle mass after 12 weeks (De Bono et al., 2006).

At 80 days of age, normally-caged SOD1$^{G93A}$ mice male mice weighed significantly more than normally-caged female SOD1$^{G93A}$ mice. Weight is usually carefully monitored in SOD1$^{G93A}$ mice because it is an early indicator of disease (Chiu et al., 1995). Spontaneous wheel running exercise caused a significant reduction in the weight of male, but not female, SOD1$^{G93A}$ mice as compared to normally caged 80 day old SOD1$^{G93A}$ control mice (Fig 5-2*A*). This significant weight loss may be accounted for by loss of muscle mass. There was a significant reduction in MG muscle weight (Fig 5-2*B*) and a trend for the TA, EDL and SOL muscle weights to be lower in exercised male SOD1$^{G93A}$ mice (Fig 5-2*BC*). In exercised SOD1$^{G93A}$ female mice, there were no significant changes in the weight of hindlimb muscles after 40 days of voluntary exercise (Fig 5-2*A*).

### 5.3.3 Wheel running accelerates the loss of motor units in TA muscles of male SOD1$^{G93A}$ mice

Following 40 days of spontaneous wheel running exercise there was a significant reduction in the number of functional TA motor units in the male SOD1$^{G93A}$ mice, as compared to normally caged SOD1$^{G93A}$ mice (Fig 5-3*A*). In female mice, voluntary wheel running did not have a significant impact on the number of intact motor units in the TA muscle, but there was a trend for motor unit numbers to increase as compared to normally-caged mice (Fig 5-3*B*).

Previously, no gender dependent differences in motoneuron number or survival were reported in SOD1$^{G93A}$ mice that volitionally ran (Kaspar et al., 2005).

We also found no significant changes in the whole muscle twitch force in the TA muscle of either female or male SOD1$^{G93A}$ mice after wheel running (Fig 5-3 *C,D*). There were trends for the whole muscle twitch force to decrease in male SOD1$^{G93A}$ mice and for an increase in twitch force in SOD1$^{G93A}$ female mice after 40 days of spontaneous exercise. These trends paralleled the changes in motor unit numbers in the two genders as compared to non-exercised control SOD1$^{G93A}$ mice. The parallel changes showed that there was no change in the forcefulness of the motor units after the voluntary wheel running (Fig 5-3*E,F*). The distribution of TA motor unit forces is plotted in Fig 5-3*E* for males and Fig 5-3*F* for females. As seen in the cumulative frequency plots in the insets of these two graphs, the distributions are almost identical following volitional running on treadmills.

### 5.3.4 Wheel running saves motor units in the MG muscle of female SOD1$^{G93A}$ mice

The same gender-dependent effects on motor unit survival were seen in the MG muscle following 40 days of voluntary running exercise as in the TA muscle of 80 day old exercised SOD1$^{G93A}$ mice. However, the decline in the number of functional motor units at 80 days of age following chronic wheel running exercise was not significant in the male SOD1$^{G93A}$ mouse MG muscle (Fig 5-4*A*). There was a significant increase in the number of motor units in the

MG muscle female SOD1$^{G93A}$ mice that had ran on wheels as compared to the control SOD1$^{G93A}$ mice (Fig 5-4*B*). In the MG, unlike in the TA muscle, the whole muscle twitch forces did not change in parallel with the changes in the motor unit numbers; in male SOD1$^{G93A}$ mice whole muscle twitch force stayed constant (Fig 5-4*C*), despite the decline in numbers of motor units. The change in the twitch force of female SOD1$^{G93A}$ mice as compared to controls was also disproportionate to the increase in motor unit numbers seen following spontaneous long term exercise (Fig 5-4*D*).

Whole muscle force in the male MG muscle was maintained because the motor units that remained following 40 days of voluntary running activity were more forceful than in the caged control SOD1$^{G93A}$ mice (Fig 5-4*E*). The parallel shift in the cumulative frequency plot in the wheel running versus caged animals illustrated in the inset of Fig 5-4*E* and Fig 5-4*F* showed that all of the surviving motor units enlarged in the MG muscles of both male and female SOD1$^{G93A}$ mice that have run for 40 days. Motor unit force is determined by the number of muscle fibers innervated by each motoneuron (the innervation ratio; IR) and the forcefulness of the muscle fibers. In the mouse muscle the force produced per muscle fiber is proportional to the cross sectional area (Andruchov et al., 2004). A previous study on the effects of exercise showed that muscle fiber CSA is unaffected by exercise (Veldink et al., 2003). In addition, motoneurons lose their sprouting ability after voluntary wheel running because peripheral schwann cells are unable to bridge to denervated muscle fibers (Tam and Gordon, 2003).

In the absence of compensatory changes that may increase motor unit force it is possible that exercise is preventing a decline in motor unit force associated with the preferential die-back of the largest motor axons and a decline in the specific force of the remaining innervated muscle fibers. We have previously shown that a decline in specific force is partially responsible for a decline in average motor unit force at 60 days of age in the TA muscle (Chapter 2). By the time of symptom onset, all type II muscle fibers are reduced in the amount of force they can produce per diameter of muscle fiber (Atkin et al., 2005). We hypothesize that exercise, which is known to help maintain muscle mass, may also be preventing the decline in specific force that causes a reduction in average motor unit force.

### 5.3.5 The effects of voluntary wheel running depend on gender in SOD1$^{G93A}$ mice

To ascertain that the effects of voluntary exercise were dependent upon gender, and the associated differences in the duration and length of voluntary exercise, we also enumerated motor units in the fast-twitch ankle-extensor EDL muscle and the slow-twitch ankle-flexor SOL muscle. In both SOD1$^{G93A}$ male and female mice voluntary wheel running did not cause a significant change in the number of EDL motor units as compared to normally caged controls (Fig 5-5*AB*). In the SOL muscle, the number of motor units was significantly lower in male SOD1$^{G93A}$ mice who had exercised than in normally caged control SOD1$^{G93A}$ mice (Fig 5-5*C*). Although no significant changes were associated

with voluntary wheel running in the SOL muscles of female SOD1$^{G93A}$ mice, there was a tendency for there to be more motor units in SOL muscles in the female SOD1$^{G93A}$ mice who had exercised (Fig 5-5*D*). A rise in the motor units of female exercised SOD1$^{G93A}$ mice was also seen in the EDL (Fig 5-5*B*). The forcefulness of the remaining motor units was not affected in either the SOL or the EDL muscle by voluntary wheel running exercise (Fig 5-5*E-F*).

These changes in the number of motor units are similar to what we saw in the TA and MG muscles of exercised SOD1$^{G93A}$ mice (Fig 5-3,4). Forty days of voluntary wheel running exercise saves motor units in female SOD1$^{G93A}$ mice but accelerates motor unit loss in male SOD1$^{G93A}$ mice. Motor unit loss may be influenced either by sex-related hormones, including estrogen which has antioxidant effects, or by the propensity of female mice to run farther and for longer than males when allowed free access to wheels. Indeed, Kaspar et al (2005) showed that the effect of voluntary exercise on prolonging lifespan was related to the amount of time that the mice were allowed to exercise, with the longest prolongation of survival time occurring in mice which had run 12 hours a day. We next attempted to increase the activity level in all mice, regardless of gender.

### 5.3.6 Partial denervation reduces the number of functional motor units in the TA muscle

The spinal roots that emanate from the L4 and L5 segments of the spinal cord in the mouse contribute to the sciatic nerve, and through it, provide

innervation to the ankle extensors and flexors in the hindlimb. In this second set of experiments we attempted to increase neuromuscular activity by avulsing the L4 spinal root and unilaterally reducing the number of functional motor units in the mouse hindlimbs (Fig 5-6*A*). Reducing the number of motor units innervating hindlimb muscles necessitates a compensatory increase in the activity of remaining motor units in order to maintain muscle function. In human patients with neurogenic lesions the maximal discharge rate of motor units increases as the number of motor units in the muscles decreased (Schulte-Mattler et al., 2000). We can therefore impose an increase in neuromuscular activity unilaterally in all mice, independent of gender or propensity for running on exercise wheels.

In normal, healthy animals partial denervation is also compensated for by enlargement of motor units through compensatory collateral axonal sprouting of the surviving motor units. In rodents, motor units can enlarge 5 to 7 fold to compensate for the loss of up to 80% of motor units (Tötösy de Zepetnek et al., 1992;Gordon et al., 1993). Fifty days after L4 spinal root avulsion in the wildtype mice, the whole muscle twitch force had recovered in our experiments there was no significant difference between the intact and the avulsed sides (Fig 5-6*B*). This recovery of the whole muscle twitch force was due to an increase in the forcefulness of the remaining, intact motor units (Fig 5-6*C*). Following L4 root avulsion, an increased proportion of the motor units produced forces above 10mN and an associated significant increase in the average motor unit force as compared to the control non-operated side (*inset* of Fig 5-6*C*). This increase in

motor unit force effectively compensated for the loss of a significant number of motor units following L4 spinal root avulsion. Approximately 60% of the motor units were lost from the TA muscle following the avulsion of the L4 spinal root.

### 5.3.7 Partial denervation is also compensated for in the other hindlimb muscles of WT control mice

To determine the effects of L4 spinal root avulsion at 40 days of age on other hindlimb muscles in the mouse we enumerated the number of motor units in the MG, EDL and SOL muscles in wildtype mice 50 days after the initial surgery. Approximately 40% of the motor units were lost from the MG muscle (Fig 5-7*A*), but the whole muscle twitch force was maintained after L4 spinal root avulsion, most likely through an increase in the proportion of more forceful motor units. The avulsion of the L4 spinal root did not significantly reduce the number of motor units in the EDL muscle of wildtype mice (Fig 5-7*B*), indicating that this muscle is not supplied by many motor axons that originate from the L4 segment of the spinal cord in the mouse. There was also no significant change in the whole muscle twitch force of the EDL muscle, as expected (Fig 5-7*C*). Because the EDL was not innervated by the L4 ventral root, we decided not to include this muscle in our analysis of the effects of partial denervation on motor unit numbers and properties in the $SOD1^{G93A}$ mouse model of ALS. The EDL has a similar muscle fiber type composition as its synergist, the TA, and the progression and rate of motor unit loss from the two muscles is very similar in both male and female $SOD1^{G93A}$ mice (Chapters 3-4). Our analysis of the effects of increased

activity on the neuromuscular system is therefore complete using three hindlimb muscles, the TA, MG and SOL. In the slow-twitch SOL muscle, there was a small, significant loss of motor units following the avulsion of the L4 root (Fig 5-7*E*), but no significant decline in whole muscle twitch force (Fig 5-7*F*).

### 5.3.8 Whole muscle twitch force is maintained in SOD1$^{G93A}$ mice following avulsion of L4 root

We next compared the contractile force of partially denervated muscles with the force produced by intact, control muscles of SOD1$^{G93A}$ male mice. The whole muscle force was not significantly different in the partially denervated TA (Fig 5-8*A*), MG (Fig 5-8*B*), or SOL (Fig 5-8*C*) muscles indicating that the loss of motoneurons was compensated for 50 days after the avulsion of the L4 ventral root. There was, however, a trend for a reduction in the whole muscle forces of the TA and the SOL muscles that had been partially denervated as compared to intact muscles in the 90 day old SOD1$^{G93A}$ mice. This muscle specific change in whole muscle force after L4 root avulsion may be due to differences in the capacity of motoneurons to reinnervate denervated muscles in the SOD1$^{G93A}$ mouse.

Frey et al. (Frey et al., 2000) reported that motoneurons innervating slow twitch muscle fibers have a greater capacity to reinnervate denervated muscle fibers at 50 days of age in the SOD1$^{G93A}$ with a robust compensatory sprouting process seen in the deepest portions of the MG muscle and throughout the SOL muscle. The TA muscle has a low proportion of type I fibers (Hämäläinen and

Pette, 1993), which are innervated by the motoneurons that exhibit a robust compensatory sprouting capacity in response to denervation (Frey et al., 2000). This lack of type I fibers may cause an insufficient increase in the average motor unit force. We have previously shown that in 60 day old SOD1$^{G93A}$ mice there was only an insignificant trend for an increase in the innervation ratio in the TA muscle, despite a denervation of ~50% of the muscle fibers (Chapter 2).

### 5.3.9 Motor units do not become more forceful to compensate for partial denervation in SOD1$^{G93A}$ mice

To detect whether the motor unit forces differ in the hindlimb muscles following ventral root avulsion in the SOD1$^{G93A}$ mice we plotted the distributions of forces from the three partially denervated muscles. In the male SOD1$^{G93A}$ mouse TA muscle the motor units that remained after avulsion of the L4 root did not become more forceful to compensate for the partial denervation of the muscle (Fig 5-9*A*). This was surprising, as the whole muscle twitch force was not significantly reduced (Fig 5-8). As the motor unit force distributions are not changed in the TA muscle (Fig 5-9*A-inset*), it appears that a compensatory process other than functional motor unit enlargement is maintaining the whole muscle twitch force, as compared to the non-operated, intact SOD1$^{G93A}$ mouse TA muscle force. In the MG muscle the percentage of motor units that produce small amounts of force was reduced, and there was an associated increase in the proportion of motor units that produce forces between 1.5 and 4 mN in partially denervated SOD1$^{G93A}$ mouse MG muscles, as compared to the intact,

unoperated side. This insignificant increase is best illustrated by the cumulative frequency plot (inset of Figure 5-9*B*), where a disproportionate increase in motor unit forcefulness is seen. The muscle-specific slight increase in the forces of some motor units in the MG, but not TA, in the $SOD1^{G93A}$ mouse muscles following partial denervation through ventral root avulsion mirrors the increase in motor unit force seen in the MG of exercised $SOD1^{G93A}$ mice. Once again, it is possible that higher proportion of type I and IIA muscle fibers in the MG are responsible for the robust compensatory sprouting process that would produce enlarged motor units capable of producing greater amounts of force.

In the SOL there is no compensatory increase in the amount of force produced by the motor units after partial denervation through the avulsion of the L4 ventral root (Fig 5-9*C*). This is surprising, given that Frey et al. (2000) reported that the motoneurons innervating the SOL are capable of robust compensatory sprouting to reinnervate denervated muscles. Whether this sprouting process produces functionally competent enlarged motor units has not yet been determined. It is possible that the amount of denervation seen in the wildtype SOL after avulsion of the L4 ventral root did cause a small amount of enlargement in the remaining motor units, but the magnitude of this increase was too small to be detected using our current methods. It is also possible that compensatory collateral axonal sprouting does not result in more forceful motor units because the reinnervated muscle fibers produce less force per fiber than the muscle fibers in the control, non-operated side of the $SOD1^{G93A}$ mice. This is unlikely because the forcefulness of muscle fibers per fiber diameter does not

decline until after disease onset in the fast-twitch muscles of SOD1$^{G93A}$ mice (Atkin et al., 2005).

### 5.3.10 Avulsion of L4 ventral root confers protections upon the remaining motor units in SOD1$^{G93A}$ male mice

We next ask the question of what processes do compensate for the loss of motoneurons that normally project through the L4 ventral root in the absence of functional motor unit enlargement. The whole muscle contractile force is dependent on both the number of motor units and the forcefulness of these motor units. If increased neuromuscular activity does confer protection upon motoneurons in the SOD1$^{G93A}$ mouse model of ALS, it is possible that the remaining motor units are less vulnerable to die-back during the pre-symptomatic phase of disease. Therefore, a saving of motor units may be responsible for the maintenance of whole muscle twitch force.

The number of motor units that remain intact in the TA muscle of wildtype, control mice following avulsion of the L4 ventral root as compared to the intact, non-operated side allows us to calculate approximately how many of the motor units were removed by the identical surgery in the 40 day old SOD1$^{G93A}$ mouse model of ALS. At 40 days of age, when there is already a significant loss of motor units from the TA muscle (Chapter 2), we calculated that avulsion of the L4 ventral root removed ~60% of the motor axons that innervated TA muscle fibers (Fig 5-10*A*). If the disease progressed, and the remaining motor units (that were innervated through the L5 and other ventral roots) continued to die-back at the

same rate as in un-operated SOD1$^{G93A}$ mouse muscles, we predicted that the number of motor units would be 40% of the number found in the control, un-operated side of the animal. That is, ~8 motor units would be left in the partially denervated TA muscles of the SOD1$^{G93A}$ mouse at 90 days of age, because 20 $\pm$ 2 motor units remain intact on the control side (Fig 5-10*A*).

We found that there were 16 $\pm$ 2 motor units remaining in partially denervated (through avulsion of the L4 ventral root) TA muscles of SOD1$^{G93A}$ mouse (Fig 5-10*A*). This was double the number that we predicted if motor unit die-back continues throughout the pre-symptomatic stage of disease (Fig 5-10*A*). The number of motor units in the partially denervated muscles was not significantly different from the number of motor units in the non-operated TA muscles. This represents a significant saving of the motor units in the TA muscle of male SOD1$^{G93A}$ mice following ventral root avulsion. There was also a trend for the saving of motor units in the MG muscle of SOD1$^{G93A}$ mice, where we found a slightly higher number of motor units than predicted (Fig 5-10*B*). In the SOL muscle of the SOD1$^{G93A}$ mouse we found no saving of motor units, and number of motor units tended to be slightly lower than we predicted (Fig 5-10*C*). The little or no saving of motor units in the MG and SOL muscle, as compared to the TA muscle may be due to the amount of activity that was induced by L4 root avulsion in the hindlimbs. The TA muscle, which showed the greatest magnitude for the saving of motor units, was also the one that was the most severely denervated by the loss of 60% of the motor units. In the MG muscle, where there was a denervation of 40%, the number of motor units that remained in the

partially denervated muscle was only slightly higher than the number we predicted would remain if there was normal motor unit die-back. Finally, in the SOL where only 10% of the motor units were lost, we found that there was no saving of motor units at all.

### 5.3.11 Motor units are also saved after avulsion of the L5 root

To test whether increasing the magnitude of partial denervation in the MG and SOL muscle would protect the remaining motor units, we avulsed the L5 ventral root instead of the L4 root, with the expectation that it contains more of the motor axons that innervate the MG muscle than the L4 root. When the muscles in control wildtype mice were partially denervated through the avulsion of the L5 ventral root, ~40% of the motor units in the TA were lost. In the MG and SOL muscles ~48% and 39% of the motor units were lost as compared to the non-operated muscles, respectively (Fig 5-11). As compared to the muscles that had been partially denervated through avulsion of the L4 ventral root, the magnitude of motor unit loss was greater following L5 root avulsion in the MG and SOL muscles, but approximately a third less in the TA muscle. Using the percent of motor units lost in the wildtype mice, we have once again calculated the proportion of motor units that would be lost from 40 day old $SOD1^{G93A}$ mice as a result of L5 ventral root avulsion (Fig 5-11). By extrapolation, we can also predict how many motor units would remain at 90 days of age in the $SOD1^{G93A}$ mice, if L5 ventral root avulsion did not affect the rate of motor unit loss. Plotting the actual number of motor units left in the partially denervated $SOD1^{G93A}$ mice

muscles at 90 days of age, we find that there is a saving of motor units in the TA (Fig 5-5-11*A*), MG (Fig 5-11*B*) and SOL (Fig 5-11*C*) muscles. In all three muscles the number of motor units in the partially denervated muscles was not significantly different from the number on the intact side.

Increasing the magnitude of denervation in the MG and SOL muscles through avulsion of the L5 ventral did confer protection upon the remaining motor units. Increasing the activity of surviving, intact motor units by removing 40% of the axons innervating the SOL muscle appeared to be more effective in retarding the progressive loss of motor units than removing only 10% of the motor units. A certain percentage of motor units may have to be lost before there is a sufficient increase in the recruitment of surviving motor units that is necessary to cause activity dependent conversion. This threshold of activity levels may also help determine when motor unit loss slows during disease progression in the $SOD1^{G93A}$ mouse. Although imposing activity upon motoneurons through low frequency electrical stimulation results in conversion of muscle fibers to slower, more oxidative phenotypes after only 14 days (Delp and Pette, 1994), the small, but significant amount of loss apparent at 40 days of age in the $SOD1^{G93A}$ may not be sufficient to provoke activity dependent changes. Moreover, recent studies have shown that changes in muscle fiber phenotypes are not always paralleled by appropriate changes in the intrinsic properties of motoneurons (Gardiner, 2006).

### 5.3.12 Protection of motor units following avulsion of L4 ventral root is not gender-dependent

Previously, we attributed gender-dependent changes in the number of motor units in the hindlimb muscles of $SOD1^{G93A}$ mice following 40 days of wheel running exercises to differences in the average distance that male and female mice spontaneous ran each night. Here, we avulsed the L4 ventral root in female $SOD1^{G93A}$ to determine whether gender had any effects on motor unit survival in conditions of imposed motor unit activity. There were no differences in the number of motor units that survived following L4 ventral root avulsion in the TA (Fig 5-12*A*), MG (Fig 5-12*B*) or in the SOL (Fig 5-12*C*), indicating that increasing the neuromuscular activity through ventral root avulsion saved motor units in gender-independent manner. Despite the potent antioxidant effects of estrogen, and its ability to protect both motoneurons (Feng et al., 2004) and skeletal muscles (Persky et al., 2000) from oxidative stress, it does not appear to protect added protection to motoneurons in conditions of imposed activity.

## 5.4 DISCUSSION

The main finding of this study was that high levels of neuromuscular activity saved functional motor units in the hindlimbs of $SOD1^{G93A}$ mice. The magnitude of this saving effect was dependent upon the gender of the mice and the type of activity that was imposed upon the hindlimb muscles. Female mice run ~40% farther and longer than males when given *ad libitum* access to exercise wheels (De Bono et al., 2006). Exercise is also known to have a dose-dependent effect on disease progression in the $SOD1^{G93A}$ mouse model of ALS (Mahoney et al., 2004;Veldink et al., 2003), with the most beneficial effects of exercise occurring when mice are allowed to volitionally exercise for ~12 hours a day (Kaspar et al., 2005). To establish that the amount of exercise, and not other gender related differences, caused the motor unit saving in female $SOD1^{G93A}$ mice we induced high levels of daily activity in three hindlimb muscles. Activity was experimentally imposed by avulsing one of the main ventral roots that supplied the MG, TA and SOL muscles in order to reduce the number of intact functional motor units by 10 to 60% (Fig 5-6 to Fig 5-7). In the $SOD1^{G93A}$ mice, the motor units that remained following ventral root avulsion were less susceptible to die-back than the motor units on the unoperated side (Fig 5-10 to Fig 5-12). The protection conferred through root avulsion was influenced by the amount of partial denervation, but not by either the gender or muscle type. We hypothesize that increased neuromuscular activity may confer protection to motoneurons by causing activity-dependent conversion to slower, more oxidative phenotypes that are less susceptible to degeneration in $SOD1^{G93A}$ mice.

Ours is the first study to evaluate muscle force and enumerate functional motor units in SOD1$^{G93A}$ mice following exercise. In association with spontaneous wheel running exercise, there was a significant decline in both the body and hindlimb muscle weight of male SOD1$^{G93A}$ mice, which was correlated with lower numbers of functional motor units in some muscles as compared to normally caged control mice (Figs 5-2 to 5-5). In contrast, there was a significant saving of motor units in female SOD1$^{G93A}$ mice. As gender determines the propensity of mice to engage in spontaneous wheel running activities (De Bono et al., 2006), our findings that motor units are spared in the more active female SOD1$^{G93A}$ mice supports previous reports that spontaneous wheel running has dose-dependent effects (Kaspar et al., 2005).

The loss of functional motor units may be slowed in exercising mice because the proportion of the most vulnerable, fast-twitch motor units may be reduced. Spontaneous running has previously been shown to reduce the number of IIB muscle fibers in rats (Gallo et al 2005), which are the first to become denervated in the SOD1$^{G93A}$ mice (Chapter 2). At 60 days of age, we have shown that there is a significant reduction in the proportion of type IIB muscle fibers due to preferential denervation and also due to conversion of fibers to less fatigable IIA fibers (Chapter 2). Accelerating this conversion of motor units to slower phenotypes through exercise may explain the saving of motor units. In males, who tend to run significantly less than females (De Bono et al., 2006), it is possible that the amount of activity is not enough to produce phenotypic conversion of the muscle fibers. Motor unit loss may even be

accelerated due to exercise induced oxidative stress (Patwell et al., 2004), which $SOD1^{G93A}$ mice may not be able to effectively buffer due to mutations to the endogenous antioxidant enzyme SOD1. Female mice may be less vulnerable to damaging oxidative species produced during exercise due to the presence of the female sex hormone estrogen, which is a potent antioxidant and can protect both skeletal muscle (Feng et al., 2004) and motoneurons (Nakamizo et al., 2000) against elevations in oxidative stress.

If exercise does cause a decrease in the proportion of the most vulnerable muscle fiber phenotypes, then why is there no significant saving of motor units in all the female mouse muscles following 40 days of spontaneous exercise? In ALS, the primary deficit is in the motoneuron, and not in the muscle, and therefore for disease progression to be slowed, protection will have to be conferred upon motoneurons, as well as the muscle fibers they innervate. The motoneurons that innervate the most resilient type I fibers have a smaller soma, higher membrane resistance, lower rheobase (Gardiner, 1993;Munson et al., 1997) and a greater complement of antioxidant enzymes (Ishihara et al., 1995) than the larger motoneurons that innervate the type II fibers. Therefore, the motoneurons innervating type I fibers are more excitable, and also able to effectively buffer the increase in oxidative stress associated with elevated activity levels.

When the MG nerve is chronically stimulated, there is an activity dependent conversion of both muscle (Gordon et al., 1997), and the associated motoneurons (Munson et al., 1997) to slower phenotypes. However,

spontaneous exercise, which also causes muscle fiber type conversion (Gallo et al., 2006;Ishihara et al., 2003) does not result in similar changes in the intrinsic motoneuron properties (Gardiner, 2006). Spontaneous running exercise was found to cause a decrease in the excitability of motoneurons (Beaumont and Gardiner, 2002). If motoneurons in $SOD1^{G93A}$ behave similarly as in wildtype controls, this activity dependent change in intrinsic properties may not confer protection by causing a shift in motoneuron characteristics towards less vulnerable phenotypes. However, in the $SOD1^{G93A}$ mice, motoneurons are found to be hyperexcitable (Kuo et al., 2004), and drugs which decrease excitability such as riluzole have been found to be beneficial. Therefore, decreasing excitability through exercise related adaptive changes may explain the significant saving of motoneurons in female $SOD1^{G93A}$ mice.

In normally caged animals we previously reported that there were no gender specific effects on progressive motor unit loss from $SOD1^{G93A}$ mice (Chapter 3). In both genders, we saw an initial quick loss of motor units that eventually flattened, much like in human ALS patients (Dantes and McComas, 1991). Motor unit loss became more gradual in all muscles of $SOD1^{G93A}$ mice during the symptomatic phase of disease, and we previously attributed this slowing to activity dependent conversion of motor units. Activity dependent conversion is already evident at 60 days of age in male $SOD1^{G93A}$ mouse TA muscles (Chapter 1), by which time ~60% of the motor units have been lost during a three week period. We hypothesize that the loss of more than half the motor units in the TA muscle necessitates an increase in the activation of the

remaining motor units. This compensatory increase causes the activity dependent conversion of muscles and associated motoneurons. As the rate and magnitude of motor unit loss are not affected by gender, the amount of activation required of the remaining motor units is equivalent in male and female mice, and there is no gender related differences in the slowing of motor unit loss. If our hypothesis of activity dependent conversion conferring saving upon motoneurons is correct, than accelerating the partial denervation and the associated conversion of motor units should have a saving effect.

Indeed, in the $SOD1^{G93A}$ mouse disease progression slowed following partial denervation of hindlimb muscles at 40 days of age by the avulsion of one of two ventral roots that supply the hindlimbs of mice (Fig 5-10 to Fig 5-12). The change in disease progression was not affected by either the gender of the animal, or the root that avulsed. The benefits associated with avulsion of either the L4 and L5 root were considered equal, unless avulsion of one root resulted in significantly greater partial denervation than the other. Therefore, axons in both roots were equally vulnerable to disease. However, disease could be slowed by increasing the magnitude of partial denervation; in the SOL muscle, a loss of approximately a tenth of the motor units innervating the muscle had no effect on disease progression, but a loss of ~40% caused motor unit loss to be slowed. The significant saving of motor units may be linked to an increased neuromuscular activity of the affected muscles. In order to compensate for a loss of 10-60% of the motor units in the hindlimb, the remaining motor units will have to increase their activity during postural and locomotor tasks. This contrasts with

the spontaneous running exercise, when the fastest, most fatigable motor units would only be activated during short bursts of intense exercise. The increased duration of neuromuscular activity may more closely resemble the muscle stimulation paradigm in which motor units are chronically activated by low frequency electrical stimulation resulting in significant conversion to slower phenotypes.

In addition, unlike the spontaneous running exercise, increased neuromuscular activity through electrical stimulation or partial denervation would not cause the monoaminergic locomotion system to be activated above normal levels. The release of the excitatory neurotransmitter 5-HT is associated with an increase in treadmill speed, among other locomotor tasks (Jacobs et al., 2002). It has been hypothesized that this increase in excitatory inputs necessitates that motoneurons become less excitable in order to adapt to chronic voluntary running exercises (Gardiner et al., 2006). This hypothesis also predicts that without increased monoaminergic inputs, the motoneurons would convert to slower, more excitable phenotypes.

We now propose a scheme whereby protection is conferred upon motoneurons in $SOD1^{G93A}$ mice because of adaptations to increased exercise (through hyperpolarization), or alternatively, increased activity of the lower motoneurons confers protection upon them by converting them to slower motoneurons with an increased excitability. In male mice, which only run half as far as the females on average, there would be no change in the resting membrane potential of the fast motoneurons which would be rarely recruited.

Therefore, there is no protection offered to them by exercise (Fig 5-13*B*). Indeed, motoneurons in male mice may be more vulnerable following spontaneous wheel running exercise due to the increased oxidative stress associated with contraction of skeletal muscle fibers (Patwell et al., 2004). In female mice which run for longer and farther than male mice on average, the putative decrease in membrane excitability may save motor units, which tend to be hyperexcitable in $SOD1^{G93A}$ mice (Fig 5-13*C*). The greatest saving of motor units in the $SOD1^{G93A}$ mouse is seen after activity was imposed upon the remaining motor units by partially denervating muscles. The chronic increases in activity, without changing the drive of the monoaminergic system, will cause an activity dependent conversion of both the motoneurons and the muscle fibers to slower, less vulnerable phenotypes (Fig 5-13*C*). Therefore, increasing the activity of the neuromuscular system in ALS may have some beneficial effects that may outweigh putative negative side effects, including excess oxidative stress.

The question of how much activity to prescribe ALS patients is a contentious issue, with current practice being to prescribe non-exhausting maintenance exercises that allow patients to focus their available energies on activities of daily living (Krivickas, 2003;Nau, 1997). Further studies are needed to characterize the changes in motoneuron properties in response to exercise, and to fully resolve the issue of whether exercise should be prescribed to patients with ALS. Coincidentally, the most recent epidemiological studies which link an increased risk of ALS to professional football players in Italy have been

done on men (Chio et al., 2005). Our studies indicate that it is possible that men who run frequently, for short bursts of time as in during football (Bangsbo et al., 2006) may hasten motoneuron loss if they are already at risk to lose motoneurons. However, exercise does appear to have a dose dependent effect and longer periods of activity, as observed in female mice, or partial denervation are needed to produce a saving effect. The implications of our study are that increased load may save motor units in the last stages of disease, and therefore exercise should not be avoided by ALS patients. Our next efforts will focus on using electrical stimulation to confer slow motoneuron properties on the remaining motor units in SOD1 mice, as per the study of Munson et al (1997).

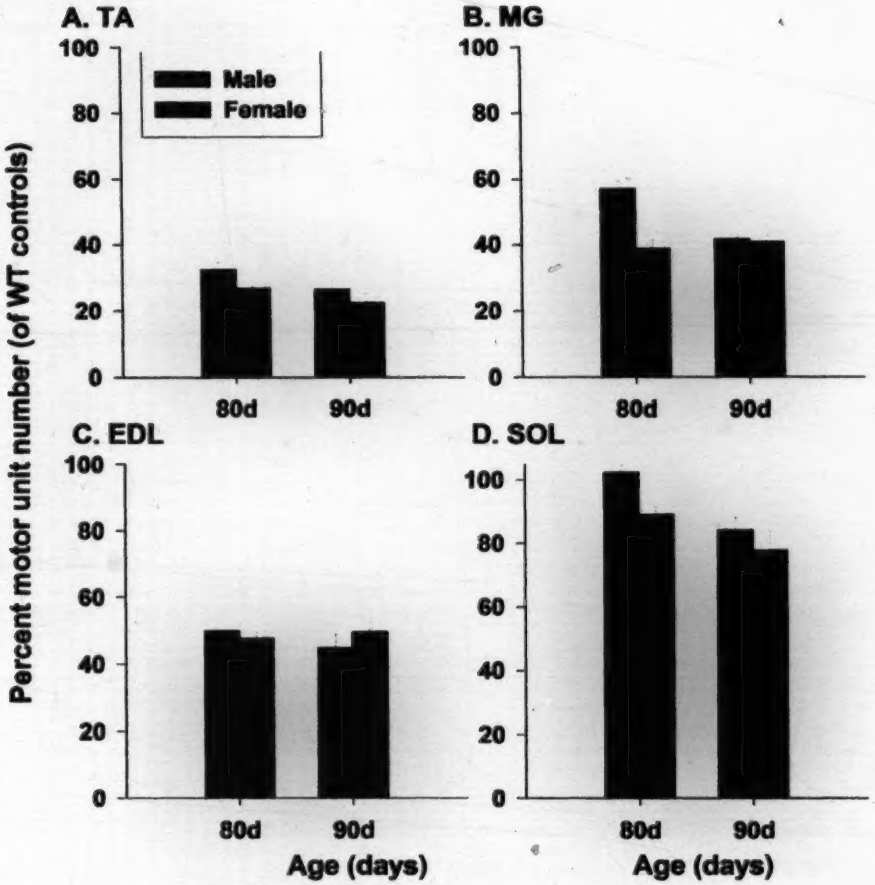


**Figure 5-1** Motor unit loss was dictated by muscle type and not gender in the hindlimb muscles of the $SOD1^{G93A}$ mice. By 80 days of age, ~10 days before the onset of overt hindlimb weakness, ~65% of the TA motor units *(A)*, 50% of the MG motor units *(B)* and 50% of the EDL motor units *(C)* have been lost in both male and female $SOD1^{G93A}$ mice as compared to wildtype controls. In comparison to these fast-twitch muscles, less than 5% of the motor units in the slow-twitch SOL muscle *(D)* have been lost prior to the onset of symptoms. There was a significant decline in the number of motor units in the SOL muscle from 80 to 90 days of age, coincident with the development of overt symptoms. However, the numbers of motor units in the fast-twitch muscles (except the male MG muscle) do not change in the interim. This plateau in the number of motor units in the fast-twitch muscles continued until end-stage disease (Chapter 3). We have hypothesized that the plateu of motor unit loss occurs due to the activity dependent conversion of the surviving motor units to less susceptible slow-twitch phenotypes (Significance is denoted by asterixes; ** indicates $p<0.01$, and * indicates $p\leq 0.05$.

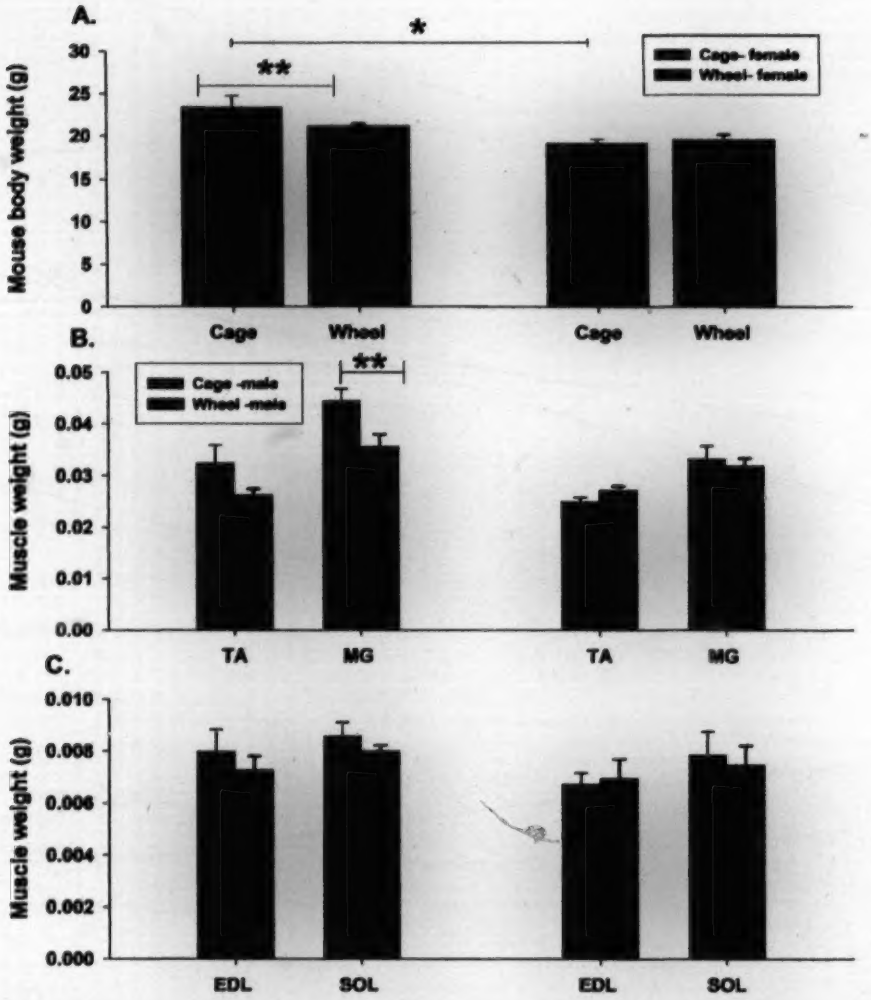


**Figure 5-2** Voluntary wheel running for 40 days caused a reduction in the weight of male SOD1$^{G93A}$ mice and in their hindlimb muscles. *(A)* The weight of male mice decreased significantly as compared to the control SOD1$^{G93A}$ mice who had been housed in normal cages without access to running wheels. Female control SOD1$^{G93A}$ mice weighed significantly less than males, and exercise did not affect the weight of female mice. *(B)* There was a trend for the weight of TA muscles to decline in the male SOD1$^{G93A}$ following exercise, and a significant decline in the weight of MG muscles following exercise. Voluntary running did not influence weight in any of the female hindlimb muscles. *(C)* There was also an insignificant trend for a reduction in the EDL and SOL muscle weights in the male, but not female SOD1$^{G93A}$ exercised mice.

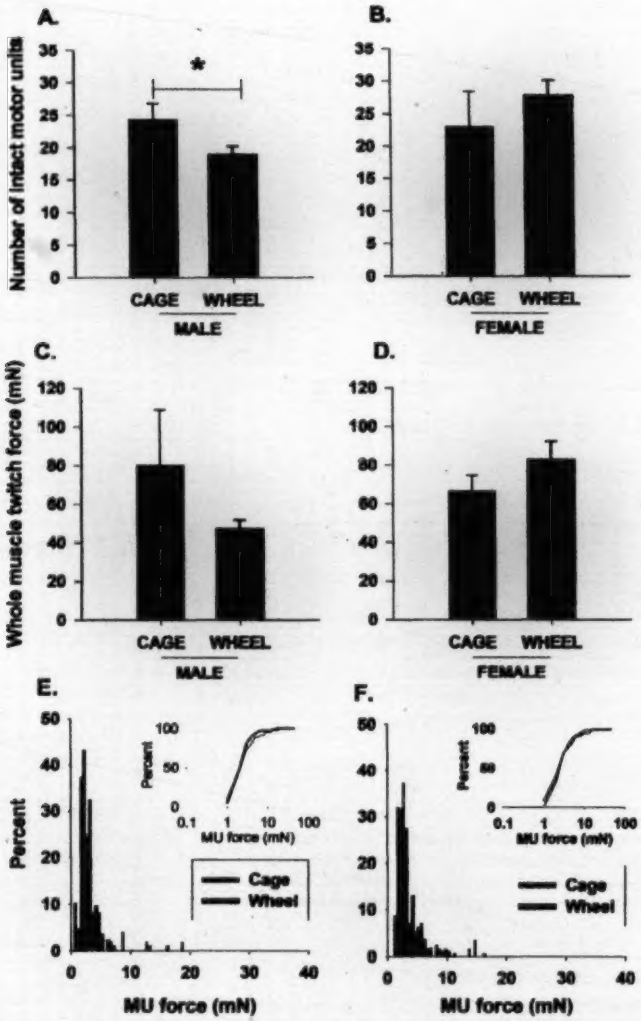


**Figure 5-3** The effect of voluntary wheel running on motor units in the TA muscles of male and female SOD1$^{G93A}$ mice. *(A)* The number of functional motor units was significantly reduced in the male SOD1$^{G93A}$ mice following 40 days of wheel running activity, as compared to non-running controls. *(B)* In female mice there was an insignificant trend for the number of TA motor units to be increased after 40 days of voluntary exercise. *(C)* In parallel with the changes in the number of functional motor units as compared to non-running controls, there was also an insignificant decline in whole muscle contractile force of male TA muscles and *(D)* a trend for increased TA muscle force in female mice after wheel running. The whole muscle twitch forces did not change significantly from control values because the distribution of motor unit forces remained constant in both male (*E*) and female (*F*) SOD1$^{G93A}$.

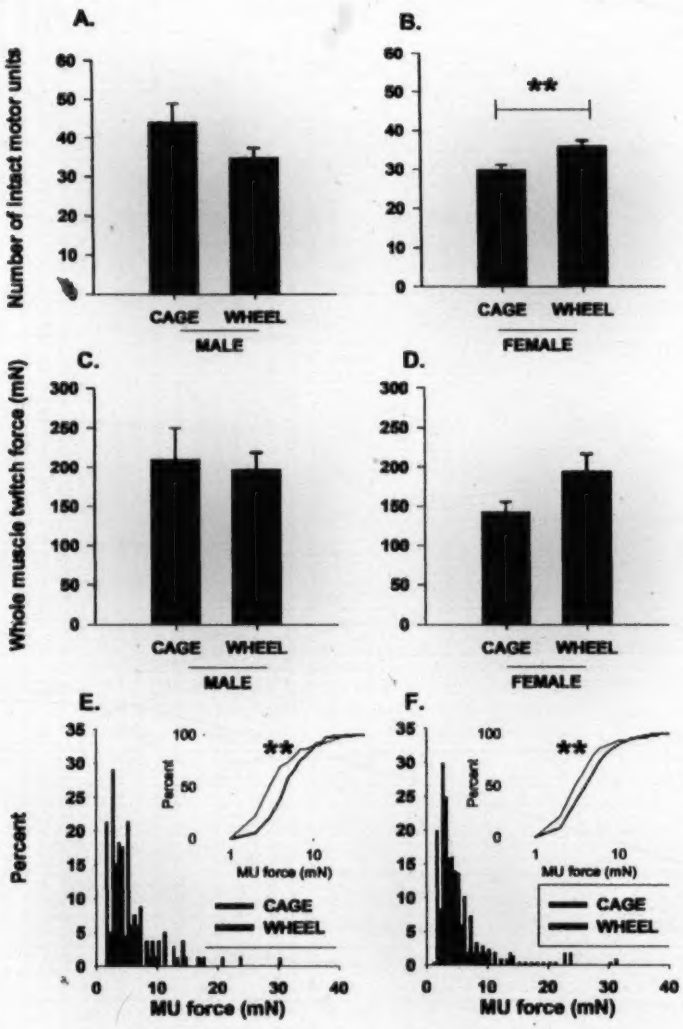


**Figure 5-4.** There is a gender-dependent saving of motor units in the SOD1$^{G93A}$ mouse MG muscle following voluntary wheel running exercise. *(A)* The number of functional motor units in the MG muscle of SOD1$^{G93A}$ male mice tended to decline, albeit insignificantly, after 40 days of running activity. *(B)* In contrast, voluntary wheel running saved motor units in the MG muscle of female SOD1$^{G93A}$ mice as compared to control non-running SOD1$^{G93A}$ mice. *(C)* Despite the trend for motor unit loss in the male MG SOD1$^{G93A}$ mouse muscle, the whole muscle twitch force did not decline as compared to control, and there was also an insignificant increase in the forcefulness of the MG muscle in the female mice *(D)*. The whole muscle contractile force did not change in parallel in the MG muscle of the SOD1$^{G93A}$ mouse because there was a significant increase in motor unit force. In both the male *(E)* and the female *(F)* SOD1$^{G93A}$ mice that had run on exercise wheels a greater percentage of motor units produced forces greater than 20mN than in the normally housed controls.

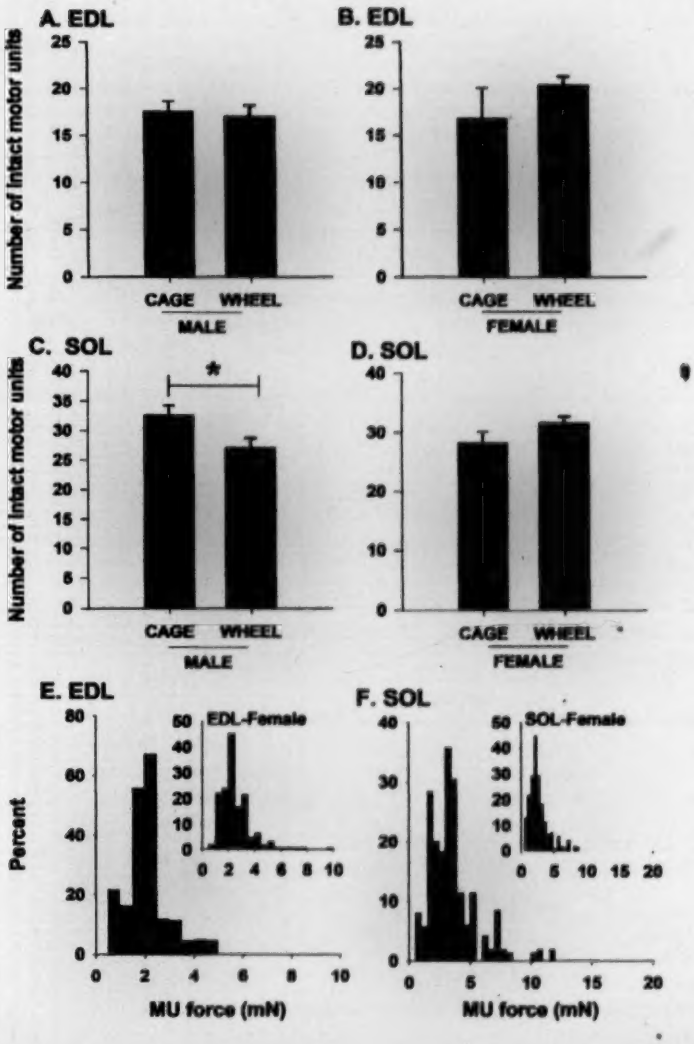


**Figure 5-5** After 40 days of voluntary wheel running activity, there is a trend for an increase in the number of motor units in both the female EDL and SOL muscles as compared to non-running control $SOD1^{G93A}$ mice. (*A*) In the male EDL muscle voluntary wheel running does not cause any change in the number of motor units. (*B*) A modest, insignificant increase in the number of motor units is seen only in the female EDL $SOD1^{G93A}$ mouse muscles after running. *(C-D)* In the SOL muscle, wheel running activity causes a significant decline in the number of motor units in the male muscles, and an insignificant increase in the number of motor units in the female SOL muscles at 80 days of age. There are no significant changes in the distribution of motor unit forces in the EDL muscles of males (*E*) or females (*E-inset*) or in the SOL muscles of males (*F*) or females (*F-inset*).

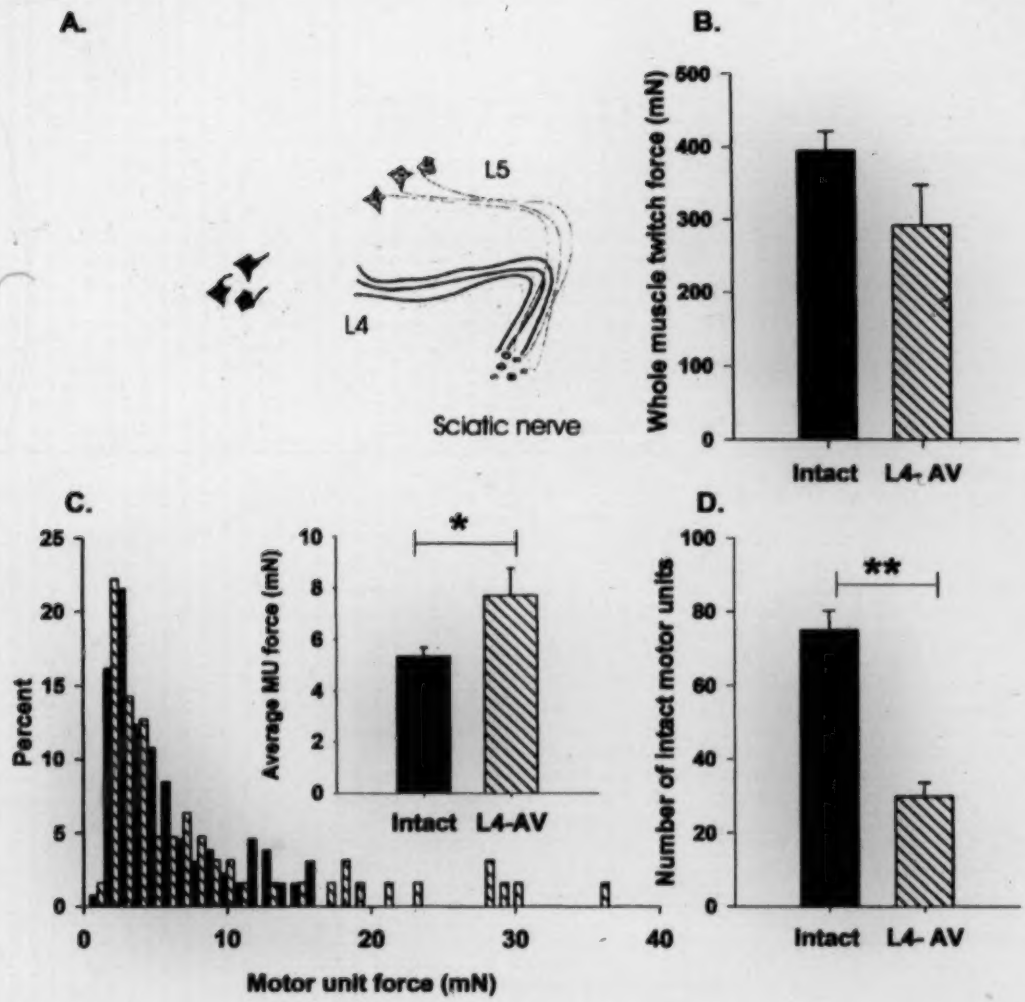


**Figure 5-6** The loss of intact motor units due to experimental avulsion of one of the main ventral roots innervating the hindlimb muscles through the sciatic nerve is compensated for by an increase in the forcefulness of the remaining motor units. *(A)* We avulsed either the L4 or the L5 ventral root on one side of the spinal cord, thus severing the connection between the motoneurons and their axons in a proportion of the motoneurons. *(B)* Despite the avulsion of the L4 ventral root, the whole muscle contractile force was not significantly reduced in the TA muscle of WT control mice 50 days after the surgery. *(C)* Whole muscle twitch force was maintained because the remaining motor units were enlarged, resulting in a significantly increased motor unit force (*inset of C*). *(D)* Thus, a loss of ~60% of the motor units, as compared to the intact side of WT control animals was compensated for.

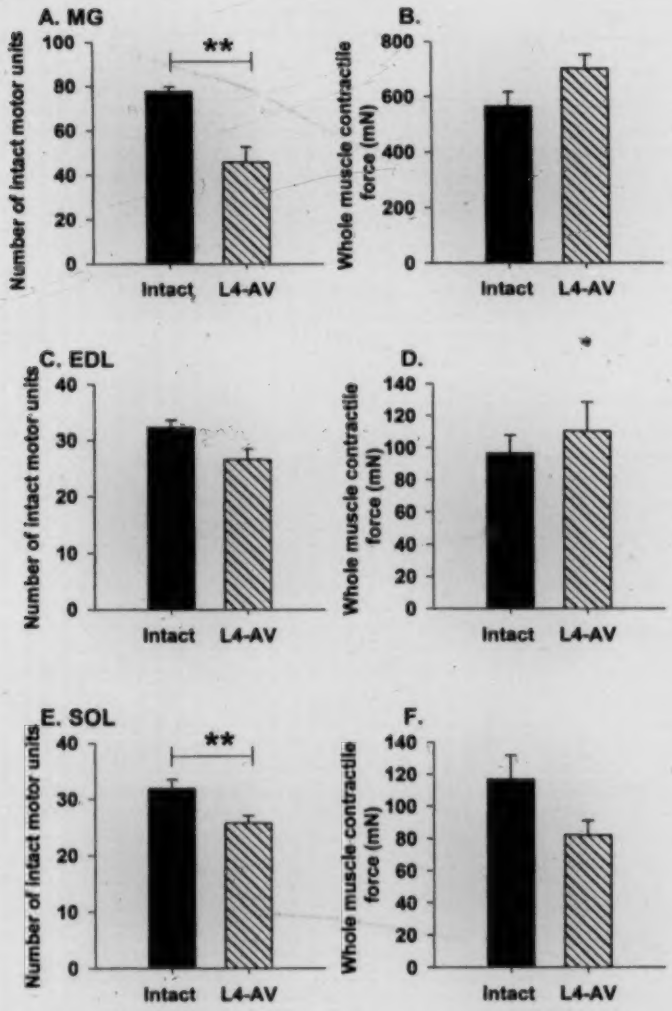


**Figure 5-7** Significant motor unit loss was also compensated for in the other hindlimb muscles of WT control mice. *(A)* In the MG muscle, avulsion of the L4 ventral root caused a 50% decline in the number of intact motor units, but the force *(B)* was not significantly different. *(C)* Avulsion of the L4 root did not cause a significantly decline in the number of EDL motor units, *(D)* and did not cause a significant change in the whole muscle force. *(E)* Motor unit numbers were decreased in the SOL muscle by ~10%, but *(F)* there was no significant change in the whole muscle twitch force.

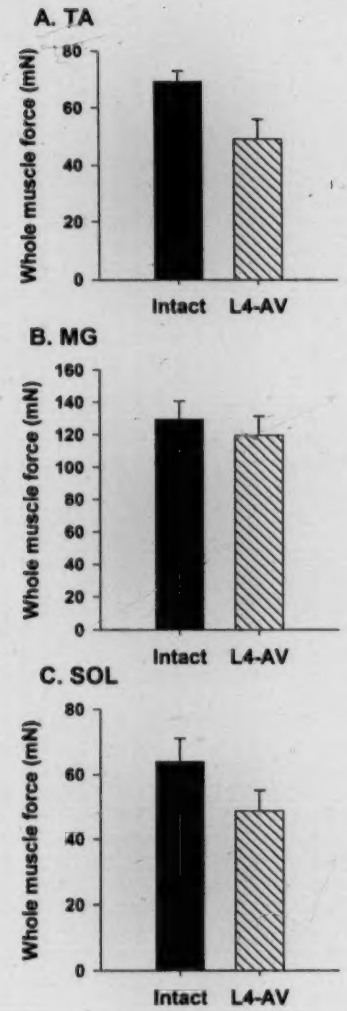


**Figure 5-8** Whole muscle twitch force recovered on the operated side of SOD1$^{G93A}$ mice 40 days after ventral root avulsion. *(A)* In the TA muscle which had approximately 60% of its innervation removed by avulsion of the L4 ventral root, whole muscle twitch force was not significantly different in SOD1$^{G93A}$ mice at 90 days of age. *(B)* In the MG and (*C*) SOL muscles the whole muscle twitch force also recovered.

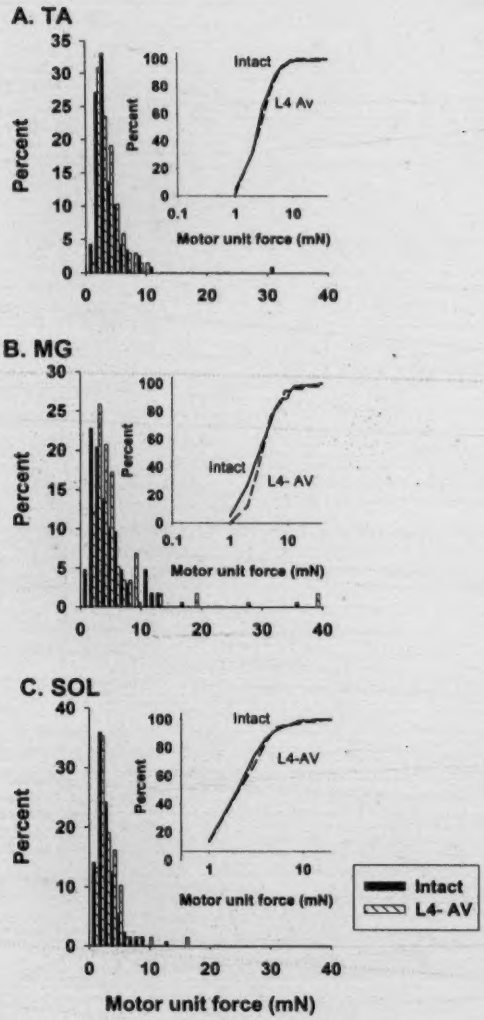


**Figure 5-9** Recovery of whole muscle twitch forces was not due to a compensatory increase in the forcefulness of surviving motor units in $SOD1^{G93A}$ mice. *(A)* The forcefulness of the surviving motor units in the TA muscle did not change in $SOD1^{G93A}$ mice. The cumulative frequency plot in the inset shows that the distribution of motor units in the intact and operated sides was identical. *(B)* There is a slight decrease in the number of motor units that produce low amounts of force in the MG muscles of $SOD1^{G93A}$ that had had L4 ventral root avulsed, as compared to $SOD1^{G93A}$ control muscles. However, the distribution of motor unit forces does not change significantly by the enlargement of a small number of motor units. *(C)* The distribution and forcefulness of motor units in the SOL muscle also stay constant on the affected hindlimbs of $SOD1^{G93A}$ mice, as compared to $SOD1^{G93A}$ mouse control SOL muscles.

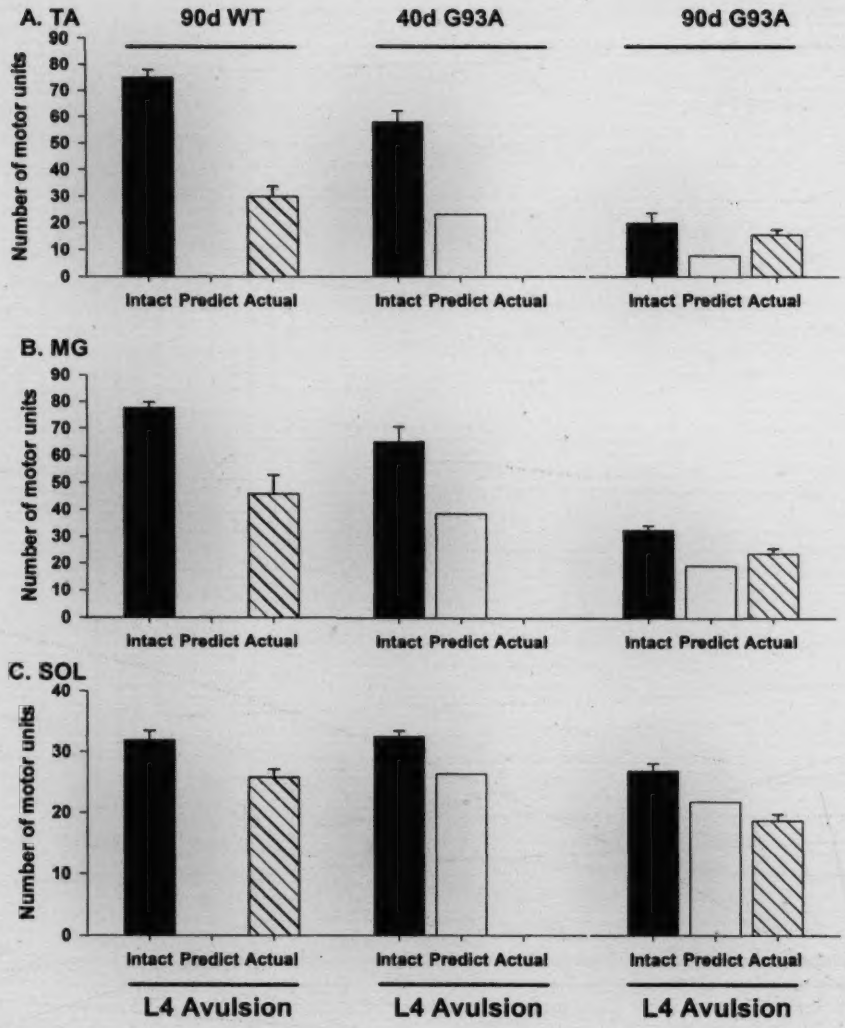


**Figure 5-10** Forty days after avulsion of the L4 ventral root the number of motor units were higher in SOD1$^{G93A}$ male mice than predicted due to protection conferred by the early increase in activity. *(A)* The avulsion of L4 ventral roots caused the loss of >60% of the motor units that innervate the TA muscle. From control data, we can predict how many motor units were lost in the SOD1$^{G93A}$ mice at the time of surgery (40 days), and then calculate the ratio of motor units that should remain at 90 days after the onset of symptoms. The number of motor units was much higher than predicted in the TA, and the MG (*B*). In the SOL, where avulsion of the L4 root affected only a small percent of the motor units, there was no saving of motor units.

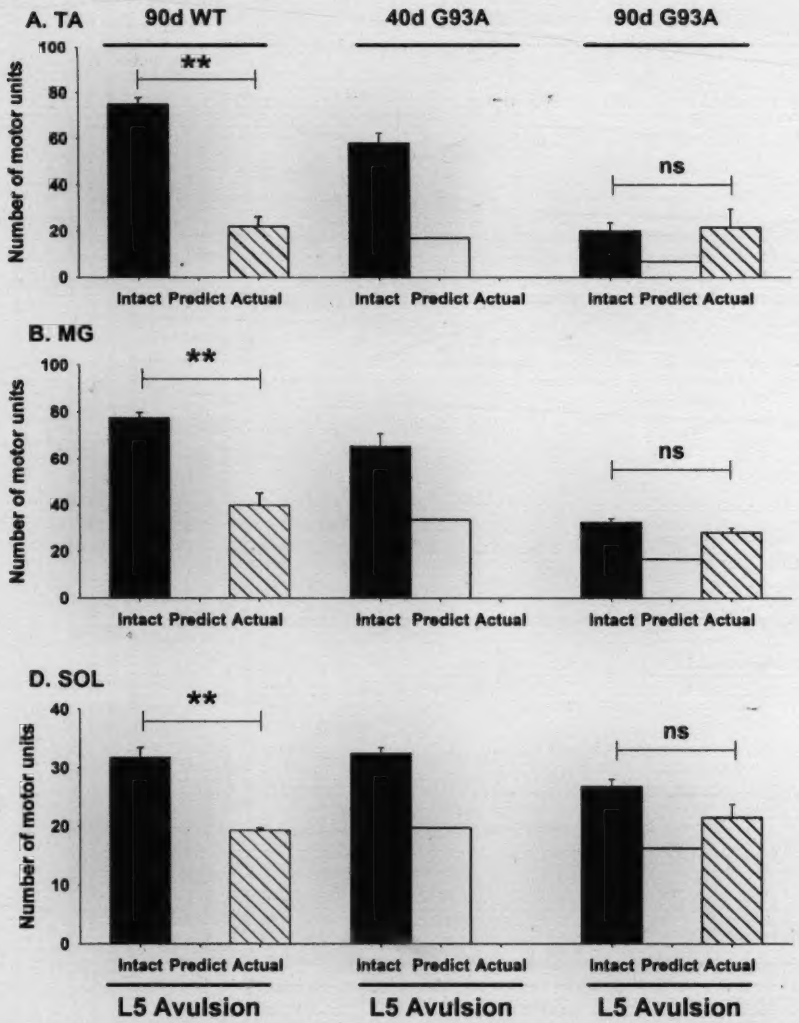


**Figure 5-11** Avulsing the L5 ventral root also conferred protection on the remaining motor units in male SOD1$^{G93A}$ mice. *(A)* The L5 ventral root supplies approximately 40% of the motor units to the TA muscle. As in the previous experiment, avulsion of the L5 root conferred protection in the TA muscle of SOD1$^{G93A}$ mice, and the number of motor units on the avulsed and non-operated side were equivelant. *(B)* Motor units were also saved in the MG muscle following L5 root avulsion, and in the SOL muscle *(C)*. In the SOL muscle, the avulsion of the L5 root caused considerably more saving than following L4 root avulsion, most likely because the magnitude of partial denervation was significantly greater.

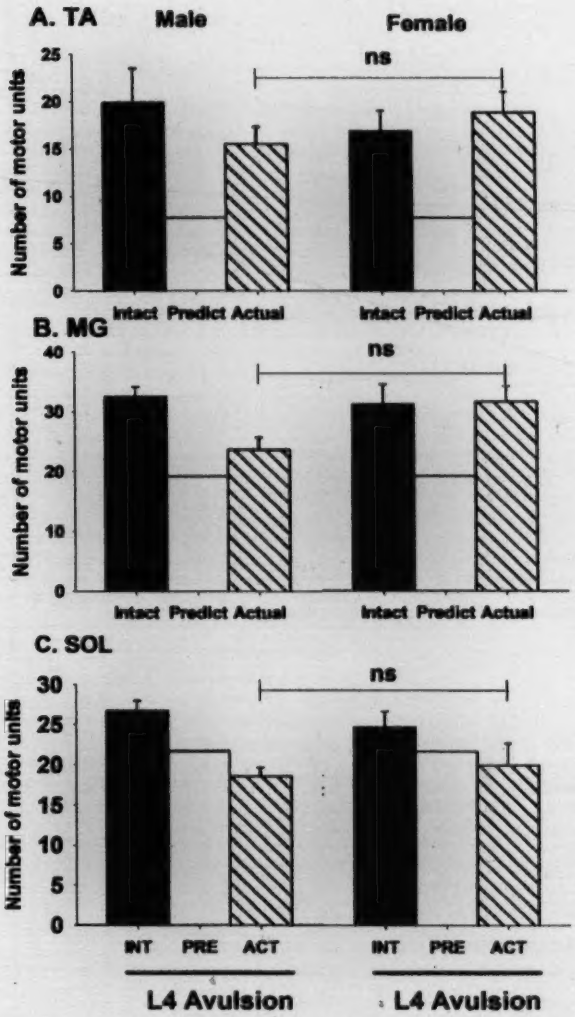


**Figure 5-12** Motor units were also saved in the female SOD1$^{G93A}$ mice after avulsion of the L4 ventral root. *(A)* In the TA muscle, avulsion of the L4 ventral root saved motor units in both male and female SOD1$^{G93A}$ mice, with a greater proportion of motor units remaining than predicted. In both males and females, the numbers of motor units on the avulsed side was not significantly different from the control, non-operated side. *(B)* In the MG the protection conferred upon motor units is greater in female than male SOD1$^{G93A}$ mice following ventral root avulsion, *(C)* and there was also some saving seen in the SOL muscle, despite the low levels of denervation achieved by the L4 ventral root avulsion.

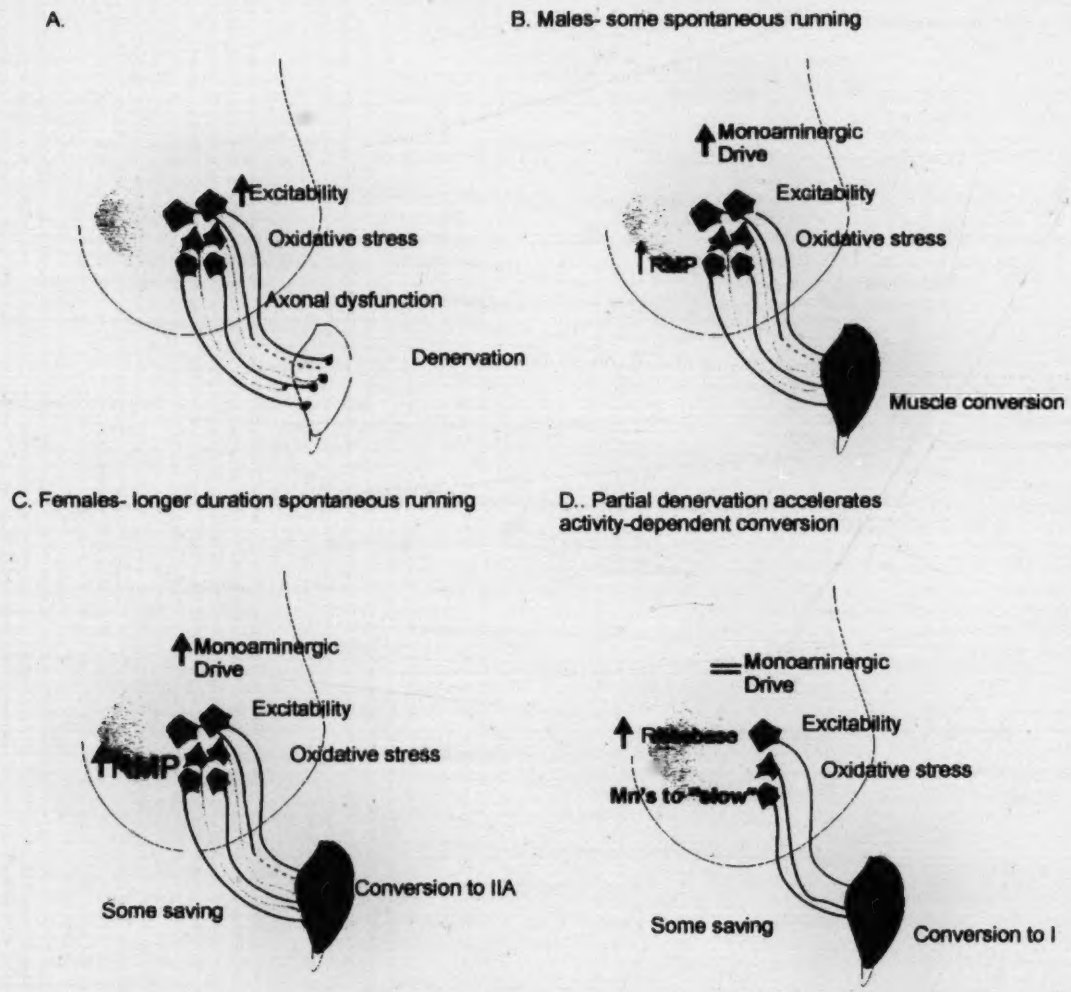


**Figure 5-13** The magnitude of the protection conferred upon motor units in SOD1$^{G93A}$ mice varies according the duration and type of activity imposed upon the neuromuscular system in the preclinical stages of disease. *(A)* Male mice run relatively shorter distances each night than female mice, resulting in some minor adaptations muscle and motoneuron phenotype. We propose that the duration of exercise is not enough to cause adaptive changes in the most vulnerable motoneurons, which innervate the fast-fatigable muscle fibers. *(B)* Changes in the resting membrane potential occur in a subset of motoneurons following long duration spontaneous exercise, as in the females. These changes, including hyperpolarization of the resting membrane potential may save motoneurons. *(C)* The greatest amount of protection is conferred by increasing neuromuscular activity through partial denervation of hindlimb muscles. Motor units are more likely to be recruited, and hence convert their properties to slower phenotypes, which causes them to be saved.

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# Chapter 6

# GENERAL DISCUSSION

## 6.1 THE EARLY, PREFERENTIAL LOSS OF MOTOR UNITS PLATEAUS IN THE SYMPTOMATIC PHASE OF DISEASE IN THE SOD1$^{G93A}$ TRANSGENIC MOUSE MODEL OF ALS

Here we report for the first time that there is an early and preferential loss of the most forceful motor units in the pre-symptomatic SOD1$^{G93A}$ mouse model of ALS. At 60 days of age, a full month before the onset of overt weakness in the hindlimbs, the numbers of intact motor units in the SOD1$^{G93A}$ mouse tibialis anterior (TA) and medial gastrocnemius (MG) muscles are reduced by ~60% (Chapter 2). This loss is not compensated for by an increase in the forcefulness of the remaining motor units (Chapter 2), despite anatomical evidence of coincident collateral axonal sprouting in denervated SOD1$^{G93A}$ mouse type I and IIA muscles (Frey et al., 2000). There is little evidence of any compensatory increase in the forcefulness of the remaining functional motor units at any time point during the disease progression in the SOD1$^{G93A}$ mouse; the loss of motor units from both fast- and slow- twitch muscles is paralleled by a decrease in whole muscle force (Chapter 3). The rate of this parallel decline in force and motor unit number is dictated by the differing proportions of slow- and fast- twitch muscle fiber types in the hindlimb muscles. Motor unit loss can be detected as early as 40 days of age in the fast-twitch TA, MG and EDL hindlimb muscles of

SOD1$^{G93A}$ mice, but there is no significant loss of motor units from the slow-twitch soleus (SOL) muscle until 90 days of age (Chapter 3). The early loss of motor units from the TA, EDL and MG muscles correspond well with previous findings of peripheral nervous system and muscle pathology at 40 days of age in the SOD1$^{G93A}$ mouse hindlimb muscles (Chiu et al., 1995). The loss of SOL motor units coincides with the onset of overt hindlimb weakness and shaking (Chapter 3). Although female mice have a significantly later onset of disease in the SOD1$^{G93A}$ mouse (Veldink et al., 2003;Trieu and Uckun, 1999), gender only had a slight effect on the disease progression (Chapter 4).

In all muscles irrespective of function, contractile properties, or gender, motor unit loss was characterized by an initial quick decline which eventually slowed until it reached a plateau during the symptomatic phase of disease (Chapters 2 to 4). A similar biphasic decline in motor unit numbers was also found in ALS patients (Dantes and McComas, 1991). We have hypothesized that the plateau in motor unit numbers occurs due to the preferential loss of the most forceful motor units because 1) the loss of the most vulnerable motor units leaves only the more resilient units and 2) early loss necessitates that the activity of the remaining motor units increase to compensate. Such an increase in motor unit activity would result in the conversion of motor units to slower, less fatigable muscle phenotypes (Gordon et al., 1997;Delp and Pette, 1994). As the slow-twitch, less forceful motor units are more resilient in SOD1$^{G93A}$ mice, activity dependent conversion would confer protection upon the remaining motor units, and thus retard motoneuron die-back. In support of this hypothesis we found that

when motor unit activity was experimentally increased by partially denervated hindlimb muscles of SOD1$^{G93A}$ mice, the remaining motor units became more resistant to loss (Chapter 5).

## 6.2 DISSOCIATION OF MOTOR UNIT SIZE AND FORCEFULNESS DUE TO PREFERENTIAL MOTOR UNIT LOSS

The early and selective loss of the most forceful motor units corresponds well with anatomical findings of preferential denervation of type IIB muscle fibers (Frey et al., 2000) and the loss of the largest axons (Fischer et al., 2004) in the SOD1$^{G93A}$ mouse. However, a decrease in the average forcefulness of surviving motor units (Chapter 2) does not agree with electromyographic (Kennel et al., 1996;Shefner et al., 2001) and anatomical findings of enlarged motor unit size (Schaefer et al., 2005). Although not all EMG studies report an increase in motor unit size in SOD1$^{G93A}$ mice (Azzouz et al., 1997) there is no evidence of a decline in size. Our experimental approach which combined anatomical and electrophysiological methods to characterize the surviving motor units at 60 days of age also revealed that despite a decline in average motor unit force, the innervation ratio remained constant (Chapter 2). Such a dissociation in motor unit size and forcefulness was previously reported only in human ALS patients (Milner-Brown et al., 1974).

This dissociation between motor unit size and forcefulness in the TA muscles of SOD1$^{G93A}$ mice occurred because the proportion of muscle fiber types changed due to the preferential denervation of the largest type IIB muscle fibers

(Chapter 2; Frey et al., 2000). A selective loss of the largest motor axons which innervate the most forceful muscle fibers (Henneman, 1985) was also reported in human ALS patients (Feinberg et al., 1999). The progressive denervation of type IIB fibers raised the proportion of smaller type IIA and IID/X fibers in the $SOD1^{G93A}$ mouse TA muscle, and hence the average muscle fiber cross sectional area (CSA) declined. The force per fiber in mammalian skeletal muscle is determined by the product of the CSA and the fiber specific force (SF; force produced per unit area). In mice the SF does not vary with fiber type (Andruchov et al., 2004;Stelzer and Widrick, 2003), and therefore a decline in CSA would cause a decline in the fiber force.

At 60 days of age the decline in fiber force was not entirely accounted for by a decline in the average muscle fiber CSA (Chapter 2). Therefore, there must have been a change in the SF of the fibers, most likely due to muscle dysfunction. In the $SOD1^{G93A}$ mouse the SF of fast-twitch muscle fibers has been shown to decline only after the onset of symptoms (Atkin et al., 2005). However, Atkin et al. (2005) did not distinguish between the different subtypes of type II fibers, and therefore a dysfunction in a specific phenotype may have been masked. Determining the SF of different muscle fiber types in human ALS patients may also help determine the mechanisms underlying the dissociation between motor unit force and size. A previous study that measured the SF of all muscle fibers did not detect any dysfunction in ALS patients (Krivickas et al., 2002).

## 6.3 PREFERENTIAL MOTOR UNIT LOSS AND ASSOCIATED REDUCTION IN WHOLE MUSCLE FORCE PRECEDE OVERT SYMPTOMS

The dissociation of motor unit size and force prevents the increase in the forcefulness of motor units in mice despite the robust compensatory sprouting response exhibited by the motor axons innervating slow-twitch muscle fibers (Frey et al., 2000). Ours is the first report of dissociation in the $SOD1^{G93A}$ mouse; previously it had been assumed that the enlargement of motor unit size in the affected hindlimbs helped compensate for early die-back and hence delay the onset of muscle weakness. We now propose that the early, uncompensated die-back of motoneurons does cause muscle weakness, but subtle and specific tests of function are required for detection. For example, at 60 days the number of motor units in the TA muscle of $SOD1^{G93A}$ mice has declined by ~60% (Chapter 2). The fast-twitch TA muscle is increasingly recruited in parallel with increases in running speed during rodent locomotion (Roy et al., 1991). Previously it has been found that the $SOD1^{G93A}$ mice cannot maintain high treadmill speeds when they are only 56 days of age (Veldink et al., 2003). This very specific decline in the maximal running speed of $SOD1^{G93A}$ mice is probably associated with the loss of TA motor units, but would not be detected by a test of gross limb function or strength.

## 6.4 INCREASING NEUROMUSCULAR ACTIVITY CONFERS PROTECTION TO MOTOR UNITS IN THE SOD1$^{G93A}$ MOUSE

Despite the inability of young SOD1$^{G93A}$ mice to maintain high running speeds (Veldink et al., 2003), voluntary running exercise has been shown to prolong their lifespan in a dose-dependent manner (Kaspar et al., 2005). If activity-dependent conversion does confer protection to surviving motor units in SOD1$^{G93A}$ mice, it is possible that exercise may accelerate the process of muscle fiber type conversion and hence confer protection upon remaining motor units. We found that there was a significant increase in the number of MG motor units in female SOD1$^{G93A}$ mice who had free access to running wheels during the pre-symptomatic phase of disease as compared to SOD1$^{G93A}$ who had been normally caged (Chapter 5). This saving effect was only seen in female SOD1$^{G93A}$ mice. There was a significant decline in the number of TA and EDL motor units in male SOD1$^{G93A}$ mice, and a trend for MG motor units to decline, as compared to muscles in normally caged mice (Chapter 5). The gender-related saving of motor units in female, but not male, SOD1$^{G93A}$ mouse hindlimb muscles may be related to the propensity of female mice to run for ~40% longer than male mice (De Bono et al., 2006).

To circumvent the gender-related differences associated with volitional exercise we experimentally increased motor unit activity by partially denervating muscles. One of the main ventral roots that innervates the hindlimb muscles (L4 or L5) was avulsed to reduce the number of intact motor units in the muscles. This reduction necessitated the increased recruitment of remaining motor units

(Patten et al., 2001), and hence increased neuromuscular activity. Fifty days after the root avulsion, the remaining motor units were less susceptible to motor unit loss in all the partially denervated hindlimb muscles of $SOD1^{G93A}$ as compared to intact controls (Chapter 5). The saving of motor units in consequence to ventral root avulsion supports our hypothesis that increases in motor unit activity save motor units. This saving effect was independent of gender.

## 6.5 EXPERIMENTAL LIMITATIONS

We now have to address some of the experimental issues that may influence our interpretation regarding the role of motor unit activity in the saving of $SOD1^{G93A}$ mouse motor. Motor unit activity was not empirically assessed in the current study. It would be possible to monitor and quantify activity levels using electromyographic (EMG) recordings of single motor units in the hindlimbs of $SOD1^{G93A}$ mice. We would expect that the remaining motor units would be increasingly recruited in parallel with the progressive die-back of the largest axons. If EMG is to be used, it is important to remember that the size of the motor units is not representative of the forcefulness of the motor units due to the changes in muscle fiber type proportions. These proportions will change with increases or decreases in neuromuscular activity. Therefore, an indirect measure of motor unit activity is the changing muscle fiber phenotype proportions. We have used immunohistochemical methods to identify muscle fiber phenotypes in 60 day old TA muscles, and consequently identified that

there is a selective denervation of type IIB fibers. A limitation of the immunohistochemical method described in Chapter 2 is that type IID/X fibers can only be identified by the absence of staining for the clone BF-35 antibodies. Using this method to identify IID/X fibers it becomes very difficult to establish which muscle fibers co-express MHC-IId(x) as well as another MHC isoform.

A limitation that affects all SOD1$^{G93A}$ mouse related ALS research is the question of how applicable the mouse model is to human forms of the disease. The SOD1- G93A mutation is present only in ~2% of all ALS patients (Rosen et al., 1993), representing a very small fraction of all ALS cases. There are also concerns as to whether the over-expression of any gene may cause some pathology in transgenic mouse models (Bergemalm et al., 2006). New mouse models which express endogenous murine mutations associated with the development of ALS-like diseases are currently being developed to address the issue of over-expression causing pathology.

## 6.6 IMPLICATIONS OF ACTIVITY-DEPENDENT MOTOR UNIT LOSS IN THE SOD1$^{G93A}$ MOUSE MODEL OF ALS

Our findings of an early and preferential loss of the most forceful motor units in the SOD1$^{G93A}$ have wide-reaching implications for the diagnosis and treatment of ALS. At present, the diagnosis of ALS is difficult as there are no readily identifiable biological markers for the disease. Here we report that the earliest sign of disease in pre-symptomatic animals is a loss of the most forceful motor units. This loss is not effectively compensated for in either SOD1$^{G93A}$ mice

(Chapters 2-3) or in human ALS patients, despite an increase in the motor unit size (Milner-Brown et al., 1974). Therefore, a test that detects dissociation between motor unit size and the average forcefulness of motor units may be useful in distinguishing ALS from other diseases that may cause distal limb weakness. The dissociation would be concomitant with a decrease in the average conduction velocity of motor axons, as a consequence of preferential large motor axon die-back (Fischer et al., 2004;Feinberg et al., 1999). Expediting the diagnosis of ALS may allow the earlier treatment of ALS patients. In the $SOD1^{G93A}$ mice therapeutic agents tend to have a better effect if they are given before the onset of disease.

## 6.7 FUTURE DIRECTIONS

The ultimate goal of ALS research to find a therapeutic treatment to effectively halt the progressive death of motoneurons. Our studies clearly demonstrate that the motoneurons which innervate postural muscles, such as the SOL, are the least vulnerable to cell death in the hindlimbs of $SOD1^{G93A}$ mice (Chapters 3-4). It is now important to determine what properties confer protection upon these motoneurons so that protection may be conferred upon other motoneuron pools. Aside from the electrical properties of motoneurons that innervate fast- and slow- twitch muscles (reviewed in the Introduction and in Burke, 1981), relatively little is known about the intrinsic and extrinsic factors that differentiate the different classes of motoneurons. As the duration afterhyperpolarization (AHP) differs (Eccles et al., 1957), it is possible that the

motoneurons have a different complement of ion channels. The excitatory and inhibitory inputs upon motoneurons may also vary, making them differentially vulnerable to degeneration of cortical motoneurons. Understanding how both extrinsic and intrinsic properties differ depending upon the contractile properties of the target muscle fibers is important in determining the factors that confer selectively vulnerability in ALS. Electrophysiological studies should therefore be conducted on both healthy and $SOD1^{G93A}$ mouse motoneurons to better understand how differences in the proportion of ion channels, extrinsic inputs and other factors influence the properties of all motoneurons. The few electrophysiological studies that have examined intrinsic motoneuron properties the $SOD1^{G93A}$ mouse have shown that the largest motoneurons are hyperexcitable in (Kuo et al., 2005;Kuo et al., 2004). Future studies should examine how the excitability of motoneurons is affected by potential therapeutic agents as well as rehabilitation protocols.

We have shown that increasing activity by partially denervating muscles saves motor units. Our hypothesis is that the saving effect is conferred by the conversion of both motoneurons and the muscle fibers they innervate to slower, more oxidative phenotypes that are less susceptible to dying back in the $SOD1^{G93A}$ mouse. To validate this hypothes the muscle fiber types and the motoneurons of $SOD1^{G93A}$ which have either voluntarily exercised, or have had either the L4 or L5 spinal root avulsed could be examined. According to the the hypothesis, such an examination would reveal a conversion of both motoneurons and muscle fibers to the slow phenotype. Imposed chronic neuromuscular

activity has been shown to alter both motoneuron (Munson et al., 1997) and muscle fiber properties (Gordon et al., 1997) to slower, more excitable phenotypes. We are now examining the muscle fiber type proportions in both $SOD1^{G93A}$ mice which had run for 40 days and in the mice in which either the L4 or L5 spinal root had been avulsed. We want to determine whether changes in muscle fiber type proportions have indeed taken place, coincident with the increase in the resilience of the motoneurons. A future study should be undertaken to examine the electrophysiological properties of the motoneurons following L4 or L5 spinal root avulsion.

## 6.8 BIBLIOGRAPHY FOR CHAPTER 6

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